Functional Role and Plasticity of $GABA_A$ Receptors in the Mouse Hippocampus

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Edith Marianne Schneider Gasser

von

Seeberg BE

Promotionskomitee

Prof. Dr. Peter Sonderegger (Vorsitz)

Prof. Dr. Beat Gähwiler

Prof. Dr. Jean-Marc Fritschy (Leitung der Dissertation)

Zürich, 2007

Para Sebastian y Martín

Table of Contents

Summary

In the mammalian brain, fast inhibitory neurotransmission is mostly mediated by $GABA_A$ receptors, which belong to the superfamily of ligand-gated ion channels. $GABA_A$ receptors form chloride channels and are the target of major drugs in clinical use, such as benzodiazepines which have sedative, anxiolytic, muscle-relaxant and anti-epileptic properties. $GABA_A$ receptor subtypes differing in subunit composition, functional properties, and pharmacological profile are differentially distributed in the brain and exhibit a synaptic or extrasynaptic distribution according to cell type and area. Therefore, $GABA_A$ receptors subtypes might regulate the activity of distinct neuronal circuits.
Studies of $GABA_A$ receptor-mediated neurotransmission have focused mainly on synaptic receptors, which are activated upon vesicular release of GABA in a transient ('phasic') manner. However, in recent years it has become evident that perisynaptic and extrasynaptic $GABA_A$ receptors, which are persistently ('tonically') activated by low GABA concentrations, play a crucial role in the regulation of neuronal excitability under physiological and pathophysiological conditions.

In this thesis, we investigated whether specific diazepam-sensitive $GABA_A$ receptor subtypes expressed in pyramidal cells and interneurons of the hippocampus are involved in phasic and tonic inhibition. As strategy, we used knock-in mice carrying diazepam-insensitive receptors generated by a histidine to arginine point mutation in the α1, α2, α3, or α5 subunit affecting a residue crucial for binding. By comparing the effects of diazepam on evoked inhibitory postsynaptic currents from pyramidal cells in different knock-in and wildtype mice, we showed that α1- and α2-$GABA_A$ receptors mediate fast phasic inhibition mostly on dendrites and soma, respectively. Furthermore, we determined the contribution of extrasynaptic α5-$GABA_A$ receptors to slow spontaneous inhibitory postsynaptic currents and to tonic inhibition by recording from triple knock-in α1α2α3 (α123) mice, in which only the α5 subunit remains sensitive to diazepam. We demonstrated that slow sIPSCs and tonic inhibition involve activation of the same pool of extrasynaptic or perisynaptic receptors containing the α5 subunit.

In interneurons, we analyzed the contribution of $GABA_A$ receptors exclusively to tonic inhibition. Mostly α1-$GABA_A$ receptors and, in certain interneurons, α2-$GABA_A$ receptors were found to mediate tonic inhibition, indicating a distinct subcellular distribution compared to pyramidal cells. Unexpectedly, interneurons in stratum oriens consistently had

a higher tonic conductance than interneurons in stratum radiatum and lacunosum-moleculare, suggesting differences in extracellular GABA concentration between these layers. Some interneurons in stratum lacunosum-moleculare completely lacked tonic conductance, indicating that tonic inhibition can also be cell-specific.

In the third part of the thesis, we studied the compensatory changes that occur in $GABA_A$ receptor expression and function in identified interneurons from mice carrying a targeted deletion of the α1 subunit gene ($\alpha1^{0/0}$). We developed a novel immunostaining protocol based on fixation of living tissue slices to allow the simultaneous detection of postsynaptic proteins and interneuron markers. We investigated the expression of $GABA_A$ receptor subunits and their synaptic or extrasynaptic location according to their colocalization with gephyrin in parvalbumin-, calretinin-, calbindin-, neuropeptide Y- or somatostatin-containing interneurons. In $\alpha1^{0/0}$ mice, synaptic α2- and α3-$GABA_A$ receptors were greatly increased in a cell-type specific manner to compensate for the loss of the α1 subunit. More synaptic receptors were evident in interneurons from $\alpha1^{0/0}$ mice, suggesting a rearrangement of GABAergic circuits, but phasic and tonic inhibition were largely unchanged, as assessed by patch clamp recordings. The amplitude of mIPSCs was reduced, suggesting a change in GABA affinity of postsynaptic receptors or presynaptic changes in vesicular GABA release. Finally, we showed that compensatory changes occurring in $\alpha1^{0/0}$ mice are sufficient to protect against kainic acid-induced seizures, indicating that GABAergic inhibition remains operative in these mutant mice.

Altogether, our results demonstrate the importance of specialized, cell-type specific $GABA_A$ receptors mediating tonic inhibition in the CA1 area of the hippocampus, and reveal that activation of extrasynaptic receptors is mostly regulated by extracellular GABA concentration, which varies across layers. The extensive morphological reorganization of $GABA_A$ receptors occurring in interneurons of $\alpha1^{0/0}$ mice highlights the plasticity of GABAergic synapses to fully compensate for the absence of a major $GABA_A$ receptor subtype. This plasticity likely reflects the potential for continuous adaptations of GABAergic transmission in the brain to maintain the function of neuronal circuits during pathological challenges.

Zusammenfassung

Im Gehirn des Säugetiers wird der grösste Teil der schnellen inhibitorischen Neurotransmission durch $GABA_A$-Rezeptoren vermittelt, die zur Superfamilie der Ligand-gesteuerten Ionenkanäle gehören. $GABA_A$-Rezeptoren bilden Chloridkanäle aus und sind das Ziel wichtiger Medikamente im klinischen Gebrauch, wie Benzodiazepine, welche sedierende, anxiolytische, muskelrelaxierende und antieptileptische Eigenschaften haben. $GABA_A$-Rezeptor-Subtypen, die sich in der Zusammensetzung der Untereinheiten, der funktionalen Eigenschaften und dem pharmakologischen Profil unterscheiden sind im Gehirn unterschiedlich verteilt und weisen eine synaptische oder extrasynaptische Verteilung auf, abhängig vom Zelltyp und Hirnareal. Daher können verschiedene $GABA_A$-Rezeptoren die Aktivität spezifischer neuronaler Schaltkreise regulieren. Studien über $GABA_A$-Rezeptor vermittelte Neurotransmission haben sich meist auf synaptische Rezeptoren gerichtet, die durch transiente (`phasische`) vesikuläre Abgabe von GABA aktiviert werden. Allerdings zeigte sich unlängst, dass perisynaptische und extrasynaptische $GABA_A$-Rezeptoren, welche dauerhaft (`tonisch`) durch niedrige GABA-Konzentrationen aktiviert werden, eine entscheidende Rolle in der Regulierung neuronaler Reizbarkeit unter physiologischen und pathophysiologischen Bedingungen spielen.

In dieser Arbeit haben wir untersucht ob spezifische, Diazepam-sensitive $GABA_A$-Rezeptor-Subtypen, die in Pyramidenzellen und Interneuronen des Hippokampus exprimiert werden, an phasischer und tonischer Inhibition beteiligt sind. Wir haben dafür 'knock-in' Mäuse mit Diazepam-insensitiven Rezeptoren benutzt, die durch eine Histidin- zu Arginin-Punktmutation der für die Bindung entscheidenden Aminosäure auf der α1, α2, α3 oder α5 Untereinheit erzeugt wurden. Durch Vergleichen der Wirkung von Diazepam auf hemmende postsynaptische Ströme von Pyramidenzellen verschiedener 'knock-in' und Wildtyp-Mäuse haben wir gezeigt, dass α1- und α2-$GABA_A$-Rezeptoren schnelle phasische Hemmung hauptsächlich auf Dendriten und Soma vermitteln. Weiter haben wir den Beitrag von extrasynaptischen α5-$GABA_A$-Rezeptoren an langsamen, spontanen, hemmenden postsynaptischen Strömen und an tonischer Hemmung durch Messungen an dreifach 'knock-in' $\alpha1\alpha2\alpha3$ (α123) Mäusen bestimmt, in welchen nur die α5 Untereinheit Diazepam-sensitiv bleibt. Wir haben gezeigt, dass langsame sIPSCs und tonische Hemmung mit einer Aktivierung desselben Pools von extra- und perisynaptischen

Rezeptoren einhergeht, die die α5 Untereinheit enthalten. In Interneuronen haben wir ausschliesslich den Beitrag von $GABA_A$-Rezeptoren an tonischer Hemmung untersucht.

Zumeist α1-$GABA_A$- und, in gewissen Interneuronen, α2-$GABA_A$-Rezeptoren vermitteln tonische Hemmung, was auf eine ausgeprägte subzelluläre Verteilung im Vergleich zu Pyramidenzellen hindeutet. Überraschenderweise hatten Interneuronen im Stratum Oriens durchwegs eine höhere tonische Leitfähigkeit als solche im Stratum Radiatum und Lacunosum-Moleculare, was auf Unterschiede in der extrazellulären GABA-Konzentration zwischen diesen Schichten schliessen lässt. In einigen Interneuronen im Stratum Lacunosum-Moleculare fehlte die tonische Leitfähigkeit vollständig, was zeigt, dass tonische Hemmung auch zellspezifisch sein kann.

Im dritten Teil dieser Arbeit haben wir die kompensatorischen Veränderungen in der Expression und Funktionalität der $GABA_A$-Rezeptoren in spezifischen Interneuronen von Mäusen mit einer Deletion der α1 Untereinheit ($\alpha1^{0/0}$) untersucht. Wir haben ein neues Protokoll für eine Immunfärbung entwickelt, das auf einer Fixierung von lebenden Gewebeschnitten basiert und den gleichzeitigen Nachweis von postsynaptischen Proteinen und `marker`-Proteinen für Interneuronen ermöglicht. Wir haben die Expression von $GABA_A$-Rezeptor-Untereinheiten untersucht und deren synaptische oder extrasynaptische Lokalisation gemäss deren Kolokalisierung mit Gephyrin in Interneuronen, welche Parvalbumin, Calretinin, Calbindin, Neuropeptide Y oder Somatostatin enthalten. In $\alpha1^{0/0}$ Mäusen waren synaptische α2- und α3-$GABA_A$-Rezeptoren auf eine zelltypische Weise stark vermehrt, um den Verlust der α1-Untereinheit zu kompensieren. In Interneuronen von $\alpha1^{0/0}$ Mäusen gab es mehr synaptische Rezeptoren, was eine Umstellung der GABAergen Schaltkreise andeutet, aber phasische und tonische Hemmung waren weitgehend unverändert, was mittels patch-clamp Messungen festgestellt wurde. Die Amplitude von mIPSCs war reduziert, was auf eine Veränderung der GABA-Affinität von postsynaptischen Rezeptoren oder presynaptischen Veränderungen vesikulärer GABA-Ausschüttung hindeutet. Schliesslich haben wir gezeigt, dass kompensatorische Veränderungen in $\alpha1^{0/0}$ Mäusen zum Schutz vor Kainat-induzierten epileptischen Anfällen ausreichen, was zeigt, dass GABAerge Hemmung in diesen Mutanten funktionsfähig bleibt.

Zusammenfassend zeigen unsere Ergebnisse die Bedeutung von spezialisierten, zelltyp-spezifischen $GABA_A$-Rezeptoren zur Vermittlung tonischer Hemmung im CA1 Bereich

des Hippokampus, und dass die Aktivierung der extrasynaptischen Rezeptoren hauptsächlich über die extrazelluläre GABA-Konzentration reguliert wird, welche über die verschiedenen Schichten variiert. Die extensive morphologische Reorganisation der $GABA_A$-Rezeptoren in Interneuronen von $\alpha 1^{0/0}$ Mäusen heben die Plastizität von GABAergen Synapsen hervor, die das Fehlen eines bedeutenden $GABA_A$-Rezeptor-Subtyps vollständig kompensieren. Diese Plastizität zeigt das Potential für kontinuierliche Anpassungen der GABAergen Transmission im Hirn, um die Funktionsfähigkeit neuronaler Schaltkreise auch während pathologischer Herausforderungen zu gewährleisten.

General Introduction

In the mammalian brain, neuronal activity is mostly regulated by GABA (γ-aminobutyric acid)-releasing inhibitory neurons. GABA is a neurotransmitter that acts on two main types of receptors: (i) ionotropic receptors called $GABA_A$ and $GABA_C$, which differ in pharmacological properties; and (ii) metabotropic receptors called $GABA_B$ (Barnard et al., 1998; Chebib and Johnston, 1999). Fast inhibitory neurotransmission in the CNS is mostly mediated by the $GABA_A$ receptors, heteropentameric chloride channels mostly composed of 2α, 2β and 1γ subunits (Tretter et al., 1997; Barnard et al., 1998; Farrar et al., 1999; Sieghart and Sperk, 2002). Most studies of inhibitory neurotransmission mediated by $GABA_A$ receptors have focused on receptors located at the synaptic cleft. However, in recent years it has become evident that GABA diffusing out the synaptic cleft can also persistently activate perisynaptic and extrasynaptic $GABA_A$ receptors, and that this tonic inhibition plays a crucial role in the regulation of neuronal excitability (Semyanov et al., 2003; Stell et al., 2003). $GABA_A$ receptors with different subunit composition are located in specific subcellular areas in defined cell types and brain areas. In pyramidal cells from the hippocampus, α5-$GABA_A$ receptors preferentially have an extrasynaptic location whereas α1- and α2-$GABA_A$ receptors are synaptically located in different subcellular compartments (Brunig et al., 2002). In interneurons, α1-$GABA_A$ receptors are abundantly expressed in both synaptic and extrasynaptic locations (Gao and Fritschy, 1994). In this thesis, we investigated whether specific $GABA_A$ receptor subtypes expressed in pyramidal cells and interneurons of the hippocampus are involved in different forms of inhibitory currents. Moreover, tonic conductance in different hippocampal interneurons from stratum oriens, radiatum and lacunosum-moleculare was compared.

In the brain, around 50% of the $GABA_A$ receptors contain the α1 subunit (Benke et al., 1994). In the hippocampal formation, α1-$GABA_A$ receptors are particularly abundant in interneurons, indicating that these receptors play a major role in interneuronal inhibitory signaling. Interestingly, deletion of the α1 subunit gene results in a viable phenotype (Sur et al., 2001; Vicini et al., 2001), implying that there are compensatory alterations in the GABAergic system to keep neuronal activity under control (Kralic et al., 2002b; Kralic et al., 2006; Ogris et al., 2006). In the hippocampus, a general upregulation of the α2, α3 and α4 subunits was observed, but the compensatory mechanisms at cellular and subcellular

level were not identified. Considering the importance of $GABA_A$ receptor plasticity in the normal functioning of the brain (Fritschy and Brunig, 2003), it is critical to determine some of the mechanisms underlying this plasticity. Therefore, we investigated how the loss of the α1 subunit is compensated for in hippocampal interneurons. We analyzed the changes in $GABA_A$ receptor subtype expression and subcellular location. Phasic and tonic inhibitory currents were also investigated to determine the functional correlates of these compensatory changes. Finally, we tested the susceptibility of the α1 knock-out mice ($\alpha1^{0/0}$) to kainic acid-induced seizures in a mouse model of temporal epilepsy (Bouilleret et al., 1999) to assess how the absence of α1-$GABA_A$ receptors affects the functioning of the inhibitory system.

1. Adult hippocampal formation and its interneuron diversity

The hippocampal formation is part of the limbic system. It is one of the most studied areas of the mammalian central nervous system because it has an easily identifiable structure and mediates major aspects of learning and memory (Eichenbaum, 2000; Scoville and Milner, 2000). The hippocampal formation is composed of three areas: the Ammon's Horn or Cornu Ammonis (CA1-CA3); also known as the "hippocampus proper", the dentate gyrus (DG), and the subiculum. The CA and DG both contain a layer of principal neurons: pyramidal cells in the CA and granule cells in the DG. Numerous local interneurons are present in each region. CA1 pyramidal cells, for example, are innervated by at least 12 distinct types of interneurons (Somogyi and Klausberger, 2005). Nevertheless, the CA1 area is probably the cortical area with the least heterogeneous neuronal population and the smallest number of extrinsic inputs, which makes it a favorite area for studying the cortical network. The laminar alignment of pyramidal cells makes it relatively easy to study the relationship between distinct cell types and the basic cortical circuit. There are five known glutamatergic inputs to CA1 pyramidal cells: from the CA3 area, the entorhinal cortex, the thalamus, the CA1 area, and the amygdala (Li et al., 1994; Somogyi and Klausberger, 2005).

Interneurons are highly diverse and functionally specialized in the mammalian central nervous system. The mapping of axonal arbors to specific domains across the dendritic trees of their targets has provided important clues to the specific functional roles carried out by various interneuron subtypes (Freund and Buzsaki, 1996). For example,

interneurons that innervate pyramidal cell somata regulate the local generation of Na^+-dependent action potentials, but inhibition arriving at dendritic locations regulates dendritic Ca^{2+}-dependent action potentials (Miles et al., 1996). The major types of hippocampal interneurons, classified according to their axonal arbors, are axo-axonic cells (Chandelier), axo-somatic cells (Basket) and axo-dendritic cells (oriens lacunosum-moleculare {O-LM} cells, bistratified, Schaffer collateral-associated and perforant pathway-associated). Some of the most useful characterizations of interneuron subtypes have been based on neurochemical content, such as the presence of calcium-binding proteins like parvalbumin (PV), calbindin (CB) and calretinin (CR), or of neuromodulators, including somatostatin (SOM), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), cholecystokinin (CCK) and nitric oxide synthase. However, neurochemically defined cells can possess different functional properties. For example parvalbumin-containing cells comprise not only basket and axo-axonic cells, but also subgroups of O-LM and bistratified cells. Therefore, parvalbumin is a marker of multiple interneuron subtypes. A summary of interneuron classes according to axonal arborization and neurochemical markers in the CA1 area is given in Table 1 and Figure 1 (McBain and Fisahn, 2001).

Characterization based on functional properties is even more problematic. Classical subdivisions were based on action potential firing patterns such as regular, irregular or clustered (Parra et al., 1998), but the generation of action potentials results from the combinations of several intrinsic voltage-gated conductances that overlap in time and have unique expression patterns throughout interneuronal sub-populations. Different expression and functional properties of voltage-gated potassium channels have provided the best insights into determinants of interneuron function. Within the hippocampus, K^+-channel subunits Kv3.1b and Kv3.2, which activate at highly depolarized potentials, are expressed in all parvalbumin-containing neurons, whereas Kv3.2 is also found in somatostatin-containing neurons. Both of these subpopulations show fast-spiking phenotypes and a large, sharp and brief afterhyperpolatization (AHP) (Freund and Buzsaki, 1996; Morin et al., 1996). The spatial distribution of channels is also an important determinant of interneuron function. The density of both Na^+ and K^+ currents is higher in dendrites of interneurons (Martina et al., 2000) so that action potentials can be initiated at dendritic sites, ensuring rapid signaling. Depending on the nature of the excitatory stimulus, action potential initiation can switch between the soma and the dendrites.

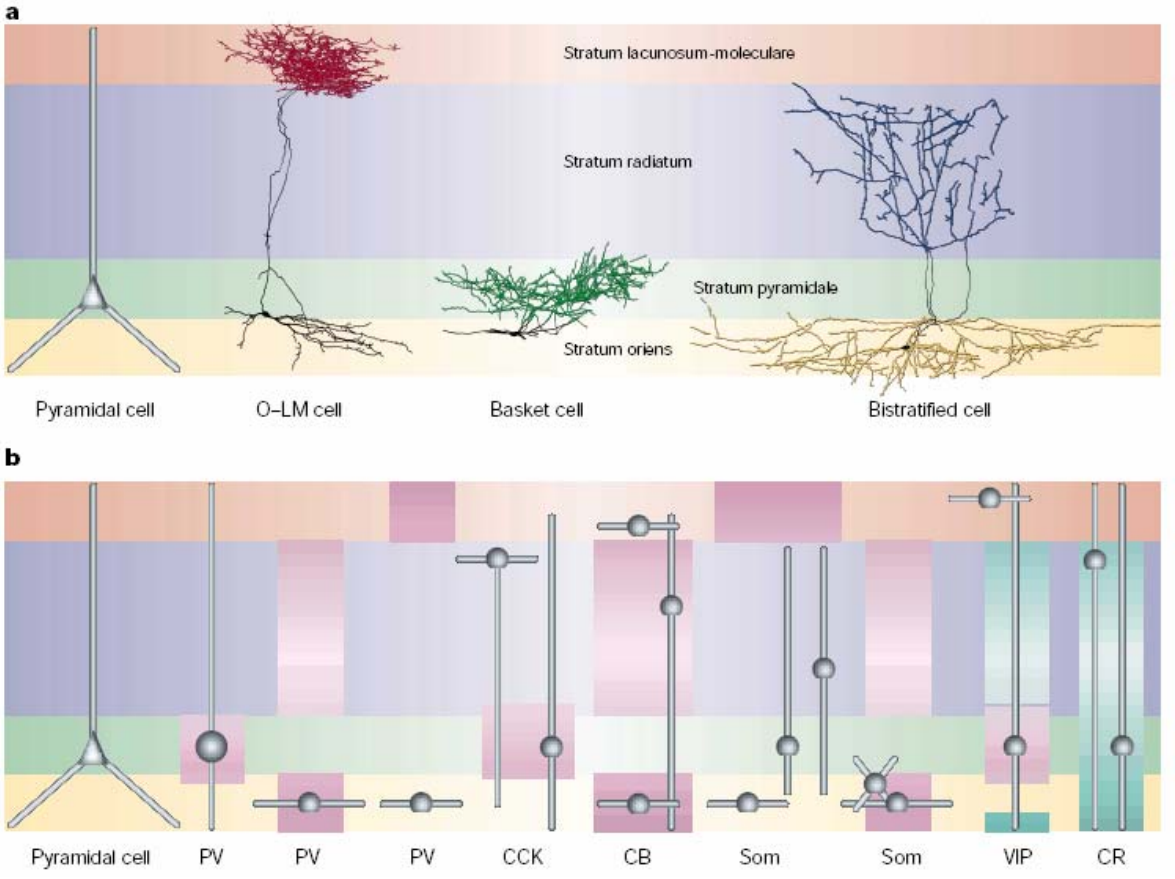


Figure 1. Domain-specific innervation of hippocampal interneurons. a) Camera lucida reconstructions of three interneurons showing the domain-specific innervation of pyramidal cells by their axons. Stratum oriens-lacunosum-moleculare (O-LM) cell with the axon (red) innervating to distal dendrites from pyramidal cells. A basket cell projects its axon (green) to the pyramidal soma and proximal dendrites. A bistratified cell sends its axon to apical (yellow) and basal (blue) dendrites. The cartoon of a pyramidal cell shows the approximate location of the basal and apical dendrites. **b)** The laminar distribution of dendritic and axonal arbors of different types of interneurons containing calcium-binding proteins and neuropeptides in the hippocampus. Circles mark the soma and lines the predominant orientation of the dendritic tree. Boxes in purple represent the laminae to which the axon of each interneuron type extends. Turquoise boxes indicate that other interneurons are targets. PV, parvalbumin; CR, calretinin; CB, calbindin; Som, somatostatin; VIP, vasoactive intestinal peptide; CCK, cholecystokinin. Figure from (McBain and Fisahn, 2001).

New molecular techniques like single-cell RT-PCR in combination with electrophysiological classification have become a powerful tool for exposing the functional diversity of GABAergic neurons at the cellular, microcircuit and systems levels. Each type of interneuron expresses a specific combination of ion channels and the pattern of expression in the surface membrane generates a type of electrical behavior (Markram et al.,

2004). A correlation map between the ion-channel genes expressed in an interneuron and its electrical phenotype provides a coefficient of correlation that gives a surprisingly highly accurate prediction of the electrophysiological parameters, given the lack of knowledge about the quantities of mRNA or protein produced by each gene (Toledo-Rodriguez et al., 2004). Correlation coefficients are independent of interneuronal morphology.

Another classification based on the ratio of excitatory and inhibitory inputs that different interneurons receive indicates whether a cell is activated in a *feedforward* (afferent volleys directly activate the inhibitory cell) or a *feedback* (recurrent collateral volleys activate the inhibitory cell) manner (Gulyas et al., 1999), which is essential for the accurate modeling of interneuron circuits.

1.1. Functional role of interneurons

Interneurons have traditionally been considered as regulators of principal neuron activity. In addition to that role, their network connectivity and intrinsic voltage-gated currents are finely tuned to permit the control of rhythmic activity of principal cells and other populations of interneurons.

Given the diversity of interneurons in the cerebral cortex, a temporally structured synchronous activity might be an organizational necessity for neuronal integration, coincidence detection, discrimination and binding of events (Singer, 1999). The most prominent rhythms are oscillations in the theta (~4–12 Hz) and gamma (~30–80 Hz) frequency bands, ultra-fast oscillations (~200 Hz) and sharp wave activity, all of which represent the synchronous discharge of principal neurons. A common feature of these different oscillations is their dependence on inhibitory interneurons (Buzsaki et al., 1983; Buzsaki, 2002). Therefore, by controlling spike timing and sculpting neuronal rhythms, inhibitory interneurons play a key role in regulating neuronal circuits and behavior.

Hippocampal network oscillations at various frequencies are correlated with certain behaviors. Theta oscillations occur during whole-body movement (Harris et al., 2002), memory tasks and rapid-eye-movement sleep (Csicsvari et al., 1999). During theta oscillations the majority of pyramidal cells in the CA1 area exhibit a very low firing rate with the highest firing probability during or shortly after the trough of the theta cycles recorded extracellularly in the pyramidal layer. Sharp wave-associated high-frequency ripples (120-200 Hz) of around 100 ms duration occur in the CA1 area of the hippocampus

during slow-wave sleep and consummatory behaviors (Buzsaki et al., 1983). Some pyramidal cells forming cell assemblies fire together, reinforcing their connections in the network by the high synchrony of discharge within a few milliseconds in each ripple cycle (Buzsaki, 1989). Gamma oscillations are not restricted to a single brain state. They occur during network activity at lower frequencies (Csicsvari et al., 2003) and also simultaneously with theta oscillations, the power of the gamma oscillations being modulated by the phase of the theta cycles.

In vivo recordings of identified interneurons in the hippocampal formation indicate that their firing pattern is strongly correlated with various network oscillations (Klausberger et al., 2003; Klausberger et al., 2004). During theta oscillations recorded extracellularly in the pyramidal cell layer, axo-axonic cells exhibit the highest firing probability around the peak of the oscillation (Klausberger et al., 2003); parvalbumin immunopositive basket cells fire at the descending phase; and bistratified cells innervating stratum radiatum and oriens and O-LM cells fire at the trough of the oscillation. Curiously, the highest probability of pyramidal cell firing coincides with maximal O-LM and bistratified cell firing. During ripple oscillations, parvalbumin-expressing basket cells and bistratified cells increase their discharge frequency. Basket cells fire at the highest amplitude of the ripple and bistratified cells fire with high frequency throughout the entire ripple episode. Axo-axonic cells slightly increase their firing activity at the beginning of the ripples, but become silent after the highest amplitude of the ripple episode, and appear suppressed even after ripple episodes. O-LM cell firing was specifically suppressed for the duration of ripple episodes. These results indicate that interneurons within a connectivity class exhibit similar firing patterns during a given network oscillation. Interneuron type-specific firing patterns point to a role in structuring the activity of pyramidal cells via their target domain, and in the accurate timing and synchronization of pyramidal cell discharge (Somogyi and Klausberger, 2005).

TABLE 1. ***Types of GABAergic interneurons in the CA1 area of the hippocampus*** (Somogyi and Klausberger, 2005)

Interneuron	**Neurochemical content**	**Cell body location**	**Cell type innervation**	**Zone of GABAergic synapse**
axo-axonic	PV	str. pyramidale	PC	axon initial segments
basket	PV	str. pyramidale	PC, PV	somata and proximal dendrites
	CCK	str. radiatum-LM	PC, CCK	somata and proximal dendrites
	CCK, VIP	str. pyramidale	PC, CCK	somata and proximal dendrites
bistratified	PV, SOM, NPY	str. pyramidale	PC, basket	proximal and distal dendrites
O-LM	PV, SOM	str. oriens	PC, INT	distal dendrites
Schaffer collateral associated	CB, CCK	str. radiatum-LM	PC, INT	apical and basal dendrites
LM-R-PP associated	CCK	str. radiatum-LM	PC	distal dendrites, dentate gyrus
LM-PP associated	CCK	str.radiatum-LM	PC	subiculum, presubiculum, dentate gyrus
neurogliaform	?	str. LM	PC	dendritic shafs and spines
trilaminar	-	str. oriens	PC	str. oriens, radiatum and LM dendrites and subiculum
back-projection	?	str. oriens	PC	somata and dendrites, CA3/ dentate gyrus
hippocampo-septal	CB, SOM	str. oriens	INT	str. oriens, str. radiatum, CA3/dentate gyrus, septum
interneuron specific I	CR	str. pyramidale	CB, CR	str. oriens and radiatum
interneuron specific II	VIP	str. radiatum-LM	CCK, VIP	str. radiatum

LM, lacunosum-moleculare; O-LM, oriens-lacunosum-moleculare; LM-R-PP, lacunosum-moleculare-radiatum-perforant path; LM-PP, lacunosum-moleculare-perforant path; PV, parvalbumin; CR, calretinin; CB, calbindin; SOM, somatostatin; VIP, vasoactive intestinal peptide; NPY, neuropeptide Y; CCK, cholecystokinin; str., stratum; PC, pyramidal cell; INT, interneuron.

2. $GABA_A$ receptor-mediated inhibitory transmission

GABA is synthesized in the cytoplasm of GABAergic cells by the irreversible decarboxylation of glutamate catalyzed by the enzyme glutamic acid decarboxylase (GAD), and is transported actively into synaptic vesicles by a vesicular carrier. When GABA is released into the synaptic cleft, it can either bind to postsynaptic $GABA_A$ receptors, postsynaptic or presynaptic $GABA_B$ receptors, and glial and neuronal GABA transporters, which take it up into presynaptic terminals or glial cells or diffuse away from the synaptic cleft and persistently activate extrasynaptic $GABA_A$ receptors (Owens and Kriegstein, 2002).

The binding of two GABA molecules to the $GABA_A$ receptor leads to a conformational change of the receptor into an open state, leading to an influx or efflux of chloride (Cl^-), depending on the membrane potential and the concentration of Cl^- in the extracellular and intracellular space. The reversal potential of the GABA current varies with the chloride equilibrium potential (E_{Cl}), but changes in K^+ and Na^+ concentrations have very little effect. The E_{Cl} is close to the resting membrane potential (around -60 mV at 20°C). In adult neurons, there is generally an influx of Cl^-, which hyperpolarizes the membrane. This hyperpolarization is the inhibitory postsynaptic potential (IPSP). When E_{Cl} is close to the resting membrane potential, no IPSP is observed even though $GABA_A$ channels are open.

Moreover, because the opening of GABA receptors decreases the membrane resistance, GABA still has an inhibitory effect on postsynaptic activity. This ‘silent inhibition` reduces the amplitude of postsynaptic depolarization and consequently prevents the generation of postsynaptic action potentials (*shunting effect*). When E_{Cl} is more positive than the resting membrane potential, a depolarization occurs. This excitatory effect of GABA has been observed in young GABAergic synapses which lack the chloride-extruding potassium chloride co-transporter KCC2 and the cytosolic carbonic anhydrase isoform CAVII and instead accumulate chloride by way of the sodium- and potassium-coupled co-transporter NKCC1 (Ben-Ari, 2002; Rivera et al., 2005). Recent studies show that the depolarizing action of GABA can also occur in some mature neurons, mostly in $GABA_A$ receptors located in axons and presynaptic terminals (Chavas and Marty, 2003; Stein and Nicoll, 2003; Szabadics et al., 2006).

In recordings with equal intra- and extracellular Cl^- concentrations to minimize *rectification* (variation of conductance as a function of membrane potential), the mean

$GABA_A$ receptor single-channel conductance is around 30 pS. The amplitude of a single-spike IPSP varies from 0.6 to 4.2 mV due to the fact that a presynaptic action potential activates, at each trial, a variable number of active zones. Approximately 300 $GABA_A$ channels are open at the peak of the single-spike IPSP. A major contributor to the amplitude variability is the large variation in the postsynaptic receptor number (Nusser et al., 1997), but at large synapses, postsynaptic $GABA_A$ receptors are not fully occupied, so amplitude variation originates from fluctuations in transmitter concentration.

The affinity of $GABA_A$ receptors for GABA is relatively low, of the order of 10-20 μM. $GABA_A$ receptor channel openings are either brief (mean duration of 2.5 ms) or in bursts (mean duration of 20-50 ms) (Bormann, 1986). The opening frequency of the $GABA_A$ channels decreases when GABA concentrations are increased (0.5 μM), leading to a reduction in GABA conductance known as $GABA_A$ receptor *desensitization.* Marked differences in desensitization kinetics have been reported between different $GABA_A$ receptor subtypes. For example, extrasynaptic α3-containing receptors desensitize very slowly (Devor et al., 2001).

2.1. Phasic (fast and slow) inhibition

Phasic inhibition refers to the transient and specific activation of $GABA_A$ receptors following brief exposure to a high concentration of GABA released from presynaptic vesicles. IPSCs are classified as fast or slow according to their amplitude and kinetics.

Fast IPSCs (Figure 2a) usually result from the rapid activation of receptors immediately beneath the release site. They peak rapidly (0.5 to 5 ms) and decay with a two-time constant ranging from 3 to 10 milliseconds. Several thousand GABA molecules are liberated into the synaptic cleft, generating a peak GABA concentration in the millimolar range (Nusser et al., 2001; Mody and Pearce, 2004) that results in the near-synchronous opening of the ion channels. The degree of receptor occupancy often varies between synapses on different neurons, and can even vary between synapses on a single neuron (Nusser et al., 1997; Hajos et al., 2000).

Slow IPSCs (Figure 2b) result from the activation of synaptic and perisynaptic receptors as a result of GABA diffusing out of the synaptic cleft (*spillover*); they peak slower than 5 ms and decay monoexponentially, ranging from 30 to 100 milliseconds. Slow IPSCs have

been described as occurring at low frequency (0.01 Hz) in pyramidal cells from the hippocampus (Pearce, 1993; Prenosil et al., 2006). Slow currents can still be considering phasic, in the sense that they are temporally related to the release event, but whether they arise from one or separate several types of GABAergic synapses has still to be investigated (Banks and Pearce, 2000; Wei et al., 2003).

The IPSC decay time depends on the length of time $GABA_A$ receptors remain in the open state. This state depends on transmitter uptake or diffusion and receptor desensitization. For small-amplitude IPSCs, the mean open time of the channels determines their decay time. However, large postsynaptic currents result from a greater presynaptic release of GABA and the slower removal of GABA from the cleft.

A major role of phasic $GABA_A$ receptor-mediated inhibition (fast and slow) is to prevent the overexcitation of neurons and thereby avoid the development of seizures, nevertheless interneurons have more complex roles, which depend crucially on the subcellular synapse location and ISPC timing. Both of these variables determine key properties of neuronal networks including: the generation of rhythmic activities (Buzsaki and Chrobak, 1995; Singer et al., 1996; Freund, 2003; Whittington and Traub, 2003; Somogyi and Klausberger, 2005); the precise coincidence detection timing at the soma of pyramidal cells and the broad detection timing on dendrites (Pouille and Scanziani, 2001); and the excitatory or inhibitory actions of GABA depolarization (Gulledge and Stuart, 2003; Williams and Stuart, 2003). Thus, minor spatial or temporal alterations in interneuronal activity may alter the way in which information is processed across neuronal networks.

2.2. Tonic inhibition

Even a low GABA concentration (micromolar range) in the extracellular space is sufficient for persistent or 'tonic' activation of $GABA_A$ receptors with high GABA affinity (Figure 2c). Tonic inhibition was first identified in cerebellar granule cells, in which application of the $GABA_A$ receptor antagonists bicuculline and gabazine not only blocked sIPSCs, but also decreased the holding current that was required to clamp cells at a given membrane potential (Kaneda et al., 1995). This reduction in input conductance was associated with a decreased number of open $GABA_A$ receptor channels (Brickley et al., 1996). Tonic activation of $GABA_A$ receptors was found to be prominent in embryonic neurons before synapse formation (Owens et al., 1999; Demarque et al., 2002). Subsequent studies

indicate that GABA-mediated tonic conductance exists in numerous adult neuron types, including: granule cells of the dentate gyrus (Nusser and Mody, 2002; Chandra et al., 2006a, b), thalamocortical relay neurons of the ventral basal complex (Porcello et al., 2003; Chandra et al., 2006b); layer V principal neurons in the somatosensory cortex (Yamada et al., 2006); CA1 pyramidal cells (Bai et al., 2001; Prenosil et al., 2006); and many inhibitory interneurons in the CA1 region of the hippocampus (Semyanov et al., 2003; Glykys et al., 2007).

The charge carried by the activation of tonically active $GABA_A$ receptors can be more than three times larger than that produced by phasic events (Nusser and Mody, 2002; Mody and Pearce, 2004; Farrant and Nusser, 2005). Tonic GABA conductance produces a shunt that affects excitability and gain control in the cells (Semyanov et al., 2003; Semyanov et al., 2004). Thus, for a given excitatory input, the size and duration of excitatory postsynaptic potentials (EPSPs) will be reduced, along with the probability that an action potential will be generated. If step-current injections of increasing amplitude are used to evoke action potentials in granule cells, blockade of tonic inhibition decreases the current required to achieve a given firing rate (Brickley et al., 1996; Hamann et al., 2002). The best demonstration that tonic inhibition is crucial for regulating neuronal excitability comes from the genetic ablation of extrasynaptic receptors. In $\alpha 6^{0/0}$ mice, for example, the missing tonic GABA current of cerebellar granule cells is replaced by a continuously active K^+ conductance of equal magnitude (Brickley et al., 2001).

2.3. Modulation of phasic and tonic inhibition

The most important difference between receptors mediating phasic and tonic inhibition is their affinity for GABA and the speed and extent of their desensitization. Receptors containing the δ subunit are involved in tonic inhibition due to their high affinity for GABA and slow desensitization kinetics (Brickley et al., 1996; Brickley et al., 2001; Nusser and Mody, 2002). Changes in extracellular GABA by modulation of GABA release and uptake determine the magnitude of tonic conductance and the kinetics of slow phasic events. In the hippocampus, it has been shown that stimulation of interneuronal firing by the glutamate-receptor agonist kainate increases the $GABA_A$ receptor-mediated tonic conductance in interneurons and pyramidal cells (Kullmann and Semyanov, 2002), whereas blockade of action-potential firing with tetrodotoxin reduces this tonic

conductance (Petrini et al., 2004). The contribution of interneurons to the generation of network oscillations might also be affected by tonic inhibition. Moreover, it has been suggested that differences in the tonic conductance of interneurons and pyramidal cells might contribute to the homeostatic regulation of phasic inhibition in pyramidal cells. Thus, blockade of tonic inhibition selectively enhances the excitability of interneurons, leading to an increase in the frequency of IPSCs in pyramidal cells (Semyanov et al., 2003).

GABA transporters, notably GAT-1, also influence ambient GABA concentrations: this transporters can rapidly redistribute between surface and intracellular compartments, and their function can be altered by phosphorylation (Quick et al., 2004) or intramolecular interactions (Hansra et al., 2004). So even if GABA release is unchanged, it is possible that ambient GABA concentrations, and thereby tonic inhibition, can be modulated by changes in uptake.

Variations in the phasic response kinetics can originate from the probabilistic nature of *quantal* transmitter release, from stochastic behavior of the receptors, or from fluctuations of the transmitter concentration in the cleft. Multivesicular release of GABA is also responsible for changes in cleft GABA concentrations (Auger et al., 1998). GABA released from two or more vesicles not only increases the peak amplitude of a phasic event, but also slows down its decay.

Another mechanism contributing to the modulation of tonic inhibition is the $GABA_B$-mediated control of GABA release by autoreceptors.

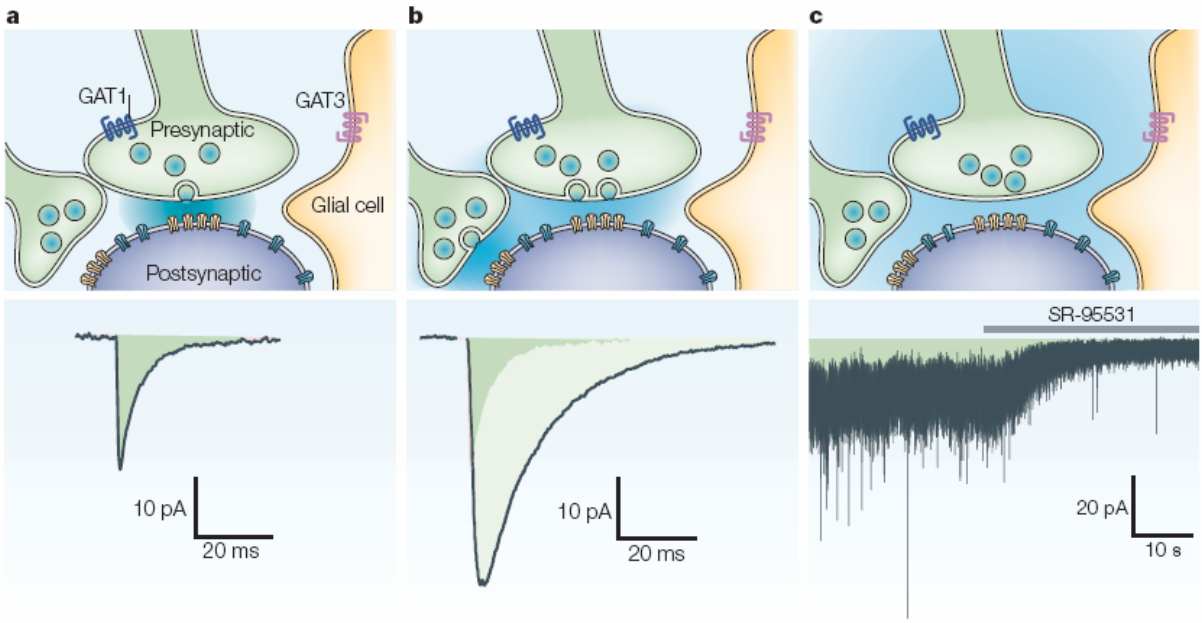


Figure 2. Modes of $GABA_A$ receptor activation. a) Phasic activation of postsynaptic receptors immediately beneath the release site (yellow). The diffuse blue shading indicates the spread of released GABA. The trace shows the average waveform of a miniature IPSC (mIPSC) recorded in the presence of the sodium blocker tetrodotoxin. The charge transfer is shaded. GAT, GABA transporter. **b)** Phasic activation of postsynaptic and perisynaptic receptors due to GABA 'spillover' (blue). The current record below shows a slow IPSC evoked by electrical stimulation. The area of the mIPSC is superimposed for comparison. c) Tonic activation of extrasynaptic receptors by a low concentration of ambient GABA, which persists despite GAT activity. The trace shows the tonic current that results from the stochastic opening of extrasynaptic receptors and the change in the holding current following application of the $GABA_A$ antagonist gabazine (SR-95531). The shaded area represents the charge carried by tonically active $GABA_A$ receptors. Figure from (Farrant and Nusser, 2005).

3. $GABA_A$ receptor diversity

The pronounced heterogeneity of GABAergic interneurons is paralleled by an extensive diversity of $GABA_A$ receptor subtypes. The region- and domain-specific location of these receptor subtypes offers an opportunity to gain insights into the role of defined neuronal circuits.

$GABA_A$ receptors belong to the superfamily of ligand-gated ion channels. They are hetero-oligomeric glycoproteins with a high structural heterogeneity. At least 21 subunits (α1–6, β1–3, γ1–3, δ, ε, ρ1–2, π and ρ1–3) have been revealed by cDNA cloning (Barnard et al., 1998; Bonnert et al., 1999; Sieghart and Sperk, 2002; Whiting, 2003). All subunits are similar in size (450-550 amino acids) and are strongly conserved among species. The common elements of the subunit structure include a large extracellular N-terminal

hydrophilic domain, four hydrophobic membrane-spanning segments (M1-4) and an extracellular C-terminal domain (Lovinger, 1997). $GABA_A$ receptor heterogeneity has important functional consequences because subunit composition dictates not only the biophysical properties (binding and gating) of the receptors, but also their subcellular distribution and therefore different modes of activation and different pharmacological actions (Pirker et al., 2000; Sieghart and Sperk, 2002; Farrant and Nusser, 2005). The subunit composition of $GABA_A$ receptors and their cellular and subcellular location is summarized in Table 2 (Fritschy and Brunig, 2003; Mohler et al., 2005).

As previously mentioned, most $GABA_A$ receptors are composed of 2α/2β/1γ subunits. Immunohistochemical, pharmacological and functional analyses of $GABA_A$ receptors indicate that the majority of $GABA_A$ receptors contain a single type of α- and β- subunit variant. The major $GABA_A$ receptor subtype is assembled from the subunits α1β2γ2 and is ubiquitously distributed throughout the brain, with only a few brain regions lacking this subtype (granule cell layer of the olfactory bulb, reticular nucleus of the thalamus, spinal cord motoneurons) (Pirker et al., 2000; Fritschy and Brunig, 2003; Mohler et al., 2005). Receptors containing the α2 and α3 subunits are less abundant and are co-expressed frequently with the β3 and γ2 subunits. α2-containing receptors are found in hippocampal pyramidal neurons, olfactory bulb, striatum, nucleus accumbens, septum, dentate gyrus, amygdala and hypothalamus. α3-containing receptors are strongly present in the glomerular and external plexiform layers of the olfactory bulb, inner layers of the cerebral cortex, reticular thalamic nucleus, superior colliculus, amygdala, and cranial nerve nuclei (Sieghart and Sperk, 2002). α5-containing receptors are expressed along with β1-3 and γ2 subunits (Fritschy and Mohler, 1995; Pirker et al., 2000). They are of minor abundance in the brain but are highly expressed in the hippocampus, olfactory bulb and hypothalamus. In general, most $GABA_A$ receptors co-assembled with γ2 subunits are located at synapses (Somogyi et al., 1996), as the γ2 subunits interact with the anchoring protein gephyrin for postsynaptic clustering (Essrich et al., 1998). An exception has been observed in pyramidal cells from the hippocampus, where in most of the α5-$GABA_A$ receptors, the γ2 subunit does not co-localize with gephyrin (Brunig et al., 2002; Crestani et al., 2002).

Other types of subunit like δ and ε exist in functional receptors. The δ subunit has a restricted distribution in the brain, being highly expressed in cerebellar and hippocampal granule cells, thalamus, and olfactory bulb. As δ subunits are not anchored at synapses,

they are found exclusively in extrasynaptic sites, (Nusser et al., 1998; Pirker et al., 2000; Stell et al., 2003; Sun et al., 2004). α6βδ combinations have been detected in the cerebellum, and α4βδ combinations in the thalamus (Sieghart and Sperk, 2002). The ε subunit is only expressed in the hypothalamus, thalamus and substantia nigra (Sieghart and Sperk, 2002). Finally, the ρ-subunits are expressed primarily in the retina, and correspond to the so-called $GABA_C$ receptors (Bormann, 2000).

3.1. $GABA_A$ receptor diversity in the hippocampus

In the hippocampal formation, pyramidal cells express a high level of α1, α2 and α5 subunits and a low level of α4 subunits (Figure 3). α1-$GABA_A$ receptors are located postsynaptically in the majority of somatodendritic synapses, whereas α2-$GABA_A$ receptors are particularly abundant in the axon initial segment (Fritschy et al., 1998). In the soma of hippocampal pyramidal cells, α1 and α2-$GABA_A$ receptor subtypes are also segregated into distinct synapses from two separate populations of basket cells (Nyiri et al., 2001; Klausberger et al., 2002). Fast-spiking interneurons expressing the neurochemical marker PV synapse with pyramidal cells through α1-$GABA_A$ receptors (Klausberger et al., 2002), whereas regular-spiking CCK interneurons form GABAergic synapses through α2-$GABA_A$ receptors (Nyiri et al., 2001). CB1-type cannabinoid receptors are localized presynaptically, on the terminals of CCK-positive basket cells. These receptors mediate *depolarization-induced suppression of inhibition*, a form of short-term synaptic plasticity induced by calcium concentration increases in postsynaptic cells (Diana and Marty, 2004). Therefore, endogenous cannabinoids modulate hippocampal circuits containing α2-$GABA_A$ receptors. The distribution of α1- and α2- containing receptors in synapses formed by different interneurons is further confirmed by our own work, in which the activation of segregated pathways activates either α1- or α2-$GABA_A$ receptors, distal extracellular stimulation (stratum radiatum or lacunosum-moleculare) activates α1-$GABA_A$ receptors, and proximal stimulation (stratum pyramidale or stratum oriens) mostly activates α2-$GABA_A$ receptors (Prenosil et al., 2006). α4- and α5-$GABA_A$ receptors have an extrasynaptic localization, being distributed throughout the somatodendritic compartments of pyramidal cells (Brunig et al., 2002; Chandra et al., 2006b; Glykys et al., 2007; Shen et al., 2007). Depending on the ambient GABA concentration, these receptors

are tonically activated (Scimemi et al., 2005). Thus, α5-$GABA_A$ receptors are activated under elevated GABA concentrations (Caraiscos et al., 2004), whereas at low GABA concentrations, the activation of α4-$GABA_A$ receptors predominates. Although a large pool of α5-$GABA_A$ receptors is extrasynaptic, (Serwanski et al., 2006), postembedding immunogold electron microscopy of the intact hippocampus showed that there is also a pool of α5-$GABA_A$ receptors present at GABAergic synapses in dendrites of hippocampal pyramidal cells. This suggests that the α5-$GABA_A$ receptor may play a functional role in phasic inhibition.

Immunocytochemical studies indicate that a large proportion of α1β2γ2 receptors are located on interneurons from the hippocampus (Fritschy and Mohler, 1995; Sperk et al., 1997; Pirker et al., 2000). All PV-positive interneurons and 50% of CR-positive neurons contain the α1 subunit, whereas CB interneurons are devoid of the α1 subunit. Similarly, most interneurons positive for NPY, and a subset of SOM-positive cells, contain the α1 subunit in contrast to CCK- and VIP-containing cells, which lack the α1 subunit (Gao and Fritschy, 1994). The cell-specific expression of $GABA_A$ receptors containing the α1 subunit among subsets of hippocampal interneurons points to a pronounced functional specialization of these cells and supports the conclusion that disinhibition may be of major functional relevance in regulating the balance between excitation and inhibition in hippocampal circuits. Contrary to pyramidal cells, the α1 subunit appears to have a mostly extrasynaptic distribution, which contributes to tonic inhibition (Glykys et al., 2007). Other α subunits are poorly expressed in CA1 interneurons, with the α3 and α5 subunits being virtually absent.

It is important to note that the expression of $GABA_A$ receptor subunits in rodents does not necessarily correspond to that in humans. For example, the α3 subunit is virtually absent in rodent hippocampus but is strongly expressed in the CA1 area of the human hippocampus (Loup et al., 2000).

TABLE 2. ***$GABA_A$ receptor subtypes*** (Fritschy and Brunig, 2003; Mohler et al., 2005)

Subunit composition	Regional and neuronal localization	Subcellular localization
$\alpha_1\beta_2\gamma_2$	Cerebral cortex (layers I-VI, selected interneurons and pyramidal cells); hippocampus (selected interneurons and pyramidal cells); amygdala, olfactory bulb (mitral cells and interneurons), thalamic relay neurons; cerebellum (Purkinje cells and granule cells); deep cerebellar nuclei; basal forebrain; substantia nigra pars reticulata; inferior colliculus; brainstem	Synaptic (soma and dendrites); extrasynaptic in all neurons with high expression
$\alpha_2\beta_3\gamma_2$	Cerebral cortex (layers I-IV); hippocampal formation (pyramidal cells); amygdala (pyramidal cells); olfactory bulb (granule cells); striatum (spiny stellate cells); inferior olivary neurons (mainly on dendrites); hypothalamus; superior colliculus; motor neurons	Synaptic, enriched in axon initial segment of cortical and hippocampal pyramidal cells
$\alpha_3\beta_n\gamma_2$	Cerebral cortex (pyramidal cells in layers V and VI); hippocampus (some hilar cells); amygdala; olfactory bulb (tufted cells), thalamic reticular neurons; cerebellum (Golgi type II cells); medullary reticular formation; inferior olivary neurons; superior colliculus; brainstem; spinal cord; medial septum; basal forebrain cholinergic neurons; raphé and locus coeruleus (serotonergic and catecholaminergic neurons)	Mainly synaptic, including some axon initial segments; extrasynaptic in inferior olivary neurons
$\alpha_4\beta_n\delta$	Dentate gyrus (granule cells); thalamus	Extrasynaptic
$\alpha_5\beta_3\gamma_2$	Hippocampus (pyramidal cells); olfactory bulb (granule cells, periglomerular cells); cerebral cortex; amygdala; hypothalamus; superior colliculus; superior olivary neurons; spinal trigeminal neurons; spinal cord	Extrasynaptic in hippocampus, cerebral cortex, olfactory bulb; synaptic in trigeminal nucleus, superior olivary nucleus, some dendrites in hippocampus
$\alpha_6\beta_{2,3}\delta$ $\alpha_6\beta_{2,3}\gamma_2$	Cerebellum (granule cells); dorsal cochlear nucleus	Synaptic (cerebellar glomeruli) and extrasynaptic on granule-cell dendrites and soma

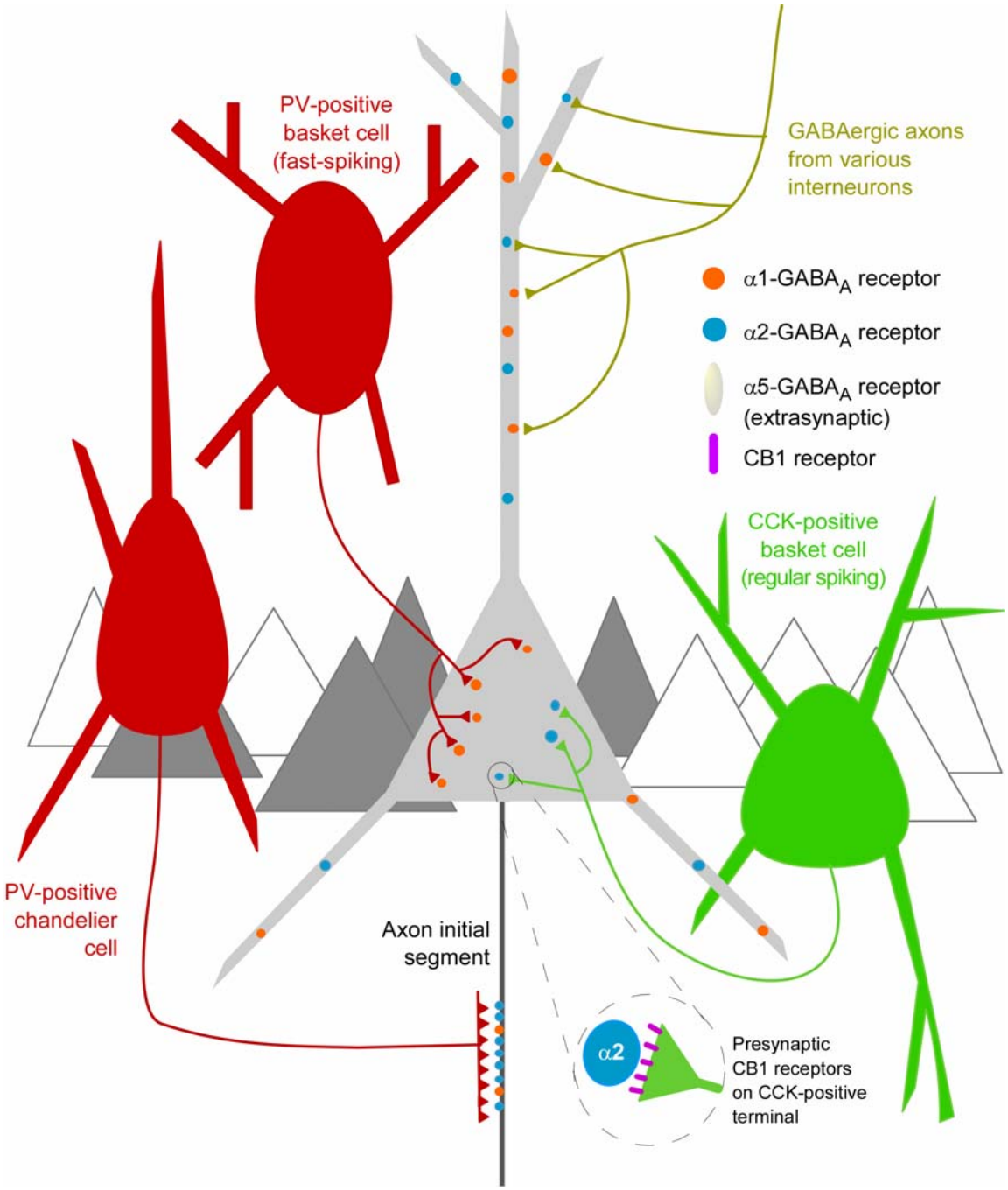


Figure 3. Diagrammatic representation of the subcellular distribution of GABA$_A$ receptor subtypes in a CA1 hippocampal pyramidal cell. CCK, cholecystokinin; PV, parvalbumin (Fritschy and Brunig, 2003)

3.2. Pharmacology of $GABA_A$ receptors

The $GABA_A$ receptor contains a variety of topographically distinct sites capable of binding clinically active substances such as benzodiazepines (sedatives, anxiolytics, hypnotics, muscle relaxants, and anticonvulsants), barbiturates (hypnotic and anticonvulsant), neurosteroids (anxiolitic, sedative, anaesthetic) and ethanol. These substances are allosteric agonists at the $GABA_A$ receptor and reversibly potentiate total $GABA_A$ currents. The structural heterogeneity of $GABA_A$ receptors also leads to pharmacological heterogeneity. Classical benzodiazepines such as diazepam, flunitrazepam and clonazepam bind at the interface between the $\alpha_{1/2/3/5}$ and γ_2-subunits (Pritchett et al., 1989; Puia et al., 1992; Sigel et al., 1998; Mohler, 2006). Benzodiazepines increase the opening frequency of the $GABA_A$ receptor and decrease the mean closed time. The different benzodiazepine actions are mediated by specific α-$GABA_A$ receptors (Table 3). For instance, the sedative and amnesic action of diazepam is mediated by α1-$GABA_A$ receptors, whereas the anxiolytic effect is mediated by α2-$GABA_A$ receptors (Mohler et al., 2002; Mohler, 2006). The dissection of receptor pharmacology has been achieved by generating lines of point-mutated mice as described in Section 4.

Barbiturates interact with an allosteric site distinct from that of benzodiazepines and, in contrast to benzodiazepines, the presence of a γ subunit is not necessary for them to exert their effects. Barbiturates increase the duration of each opening of the $GABA_A$ receptor. At high concentrations, barbiturates can also open $GABA_A$ channels directly, another effect that distinguishes them from the benzodiazepines.

Neurosteroids, such as allotetrahydrodeoxycorticosterone (THDOC), act preferentially on diazepam-insensitive receptors containing the $\alpha_{4/6}$ and δ subunits (Belelli et al., 2002; Brown et al., 2002; Wohlfarth et al., 2002), and increase both the open probability and the duration of burst openings. Either or both of these effects result in an increase of GABA currents. It is important to note that the I/V curves evoked in the presence or absence of agonists reverse at the same potential, so that the ion selectivity of the channel is unaltered (Rogers et al., 1994). Similarly, ethanol acts on receptors containing the $\alpha_{4/6}$ and δ subunits (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Hanchar et al., 2005) (Table 3).

Bicuculline, SR-95531 and picrotoxin reversibly decrease total $GABA_A$ current; they are, respectively, competitive, competitive, and non-competitive antagonists of the $GABA_A$

receptors and are potent anticonvulsants (Macdonald and Barker, 1978; Newland and Cull-Candy, 1992). $GABA_A$ receptor antagonists are useful for the experimental investigation of tonic and phasic inhibition because their function depends on the affinity of the receptors for GABA and on the conditions of the receptor activation. For example, SR-95531 at sub-micromolar concentrations selectively blocks phasic currents (Bai et al., 2001; Stell and Mody, 2002; Semyanov et al., 2003; Yeung et al., 2003), which is consistent with the underlying receptors having a lower affinity for GABA than those that mediate tonic current (Stell and Mody, 2002). The channel blocker penicillin also selectively blocks phasic currents, reflecting the low occupancy of tonic receptors (Yeung et al., 2003). Furosemide is another antagonist with selectivity for α6-$GABA_A$ receptors (Korpi et al., 1995).

4. Animal models for the dissection of $GABA_A$ receptor functions

The use of pharmacological compounds to investigate the functional role of specific $GABA_A$ receptors has limitations, because few compounds show clear subunit selectivity. Therefore genetic approaches are used. In the past years several knock-in and knock-out mice for one or more $GABA_A$ receptor subunits have been generated. Knock-in mice that carry point mutations in the amino acid responsible for the binding of modulatory drugs like diazepam have enabled the exploration of the pharmacological role of defined $GABA_A$ receptor subtypes (Rudolph et al., 1999; Low et al., 2000; Crestani et al., 2001; Crestani et al., 2002; van Rijnsoever et al., 2004) and provided a potent tool for studying the organization of inhibitory circuits at a functional level (Marowsky et al., 2004). Knock-out mice for specific $GABA_A$ receptor subtypes have not only provided important insights into the functions of $GABA_A$ receptor subtypes but also insights into the compensatory changes that may occur, which are of relevance to understanding inhibitory plasticity and interpreting a possible behavioral phenotype due to the mutation.

TABLE 3. ***$GABA_A$ receptor subtype ligands and pharmacological action*** (Fritschy and Brunig, 2003; Mohler et al., 2005; Rudolph and Mohler, 2006)

Drug	**Interaction with the $GABA_A$ receptor subunits**	**Pharmacological action**
Diazepam (BZ)	$\alpha1$	Sedative, anterograde amnesia, anticonvulsion, tolerance to sedation, ethanol potentiation
	$\alpha2$	Anxiolytic, myorelaxation, modulation of rhythmic EEG
	$\alpha3$	Anticonvulsion, myorelaxation
	$\alpha5$	Anterograde amnesia*, muscle relaxation, associative and spatial memory, tolerance to sedation
Zolpidem (BZ)	$\alpha1$	Sedative, hypnotic, anticonvulsion, EEG power reduction in non-REM sleep
Zaleplone (BZ)	$\alpha1$	Hypnotic
Indiplon (BZ)	$\alpha1$	Hypnotic
L-838 417 (BZ)	$\alpha2$, $\alpha3$, $\alpha5$ partial agonist, $\alpha1$ inverse agonist	Anxiolytic
Ocinaplon (BZ)	$\alpha2$, $\alpha3$, $\alpha5$ partial agonist, $\alpha1$ full agonist	Anxiolytic
L-655 708 (BZ)	partial inverse agonist for $\alpha5$	Memory enhancer, anxiogenic
Ethanol	$\alpha_4\beta_3\delta$, $\alpha_6\beta_3\delta$ high sensitivity; $\alpha_4\beta_2\delta$, $\alpha_6\beta_2\delta$ medium sensitivity	Sedative, anxiolytic
Neurosteroids (THDOC)	δ–containing receptors and $\alpha1$, $\alpha3$ receptors in combination with $\beta1$	Anxiolytic, sedative, anaesthetic
Intravenous anesthetics (etomidate, propofol)	$\beta3$-containing receptors	Sedative, anaesthetic
Gabodaxol	$\alpha1$ and $\alpha3$ at the GABA site	Hypnotic

* tested in $\alpha5^{0/0}$ mice; BZ, benzodiazepine site ligands.

4.1. $GABA_A$ receptor α subunit knock-in mice

Point mutations were introduced into specific diazepam-sensitive $GABA_A$ receptor α subunit genes to obtain diazepam-insensitive $GABA_A$ receptors with intact expression and physiological function, thus avoiding developmental compensatory mechanisms. A histidine residue at a conserved position (α1-H101, α2-H101, α3-H126, and α5-H105) forms a high-affinity diazepam-binding site, whereas an arginine residue (α4-R99 and α6-R100) is unable to do so. When an arginine residue replaces the histidine residue in the α1, α2, α3 and α5 subunits, recombinant receptors become diazepam insensitive (Wieland et al., 1992; Benson et al., 1998). To elucidate the involvement of defined $GABA_A$ receptor subtypes in different actions of diazepam *in vivo,* diazepam-insensitive mice [α1(H101R), α2(H101R), α3(H126R) and α5(H105R)] were generated. The respective behavioral deficit in different α subunit knock-in mice in response to diazepam treatment is attributed to the respective $GABA_A$ receptor subtype. Immunoblotting, immunohistochemistry, and immunofluorescence analysis reveal that the mutant receptor α1-3 subunits are expressed at normal levels, with unaltered regional and subcellular distributions. In α5(H105R) mice, a 30% reduction in expression of the α5 subunit in the hippocampus was observed, but the regional and subcellular localization was unchanged (Crestani et al., 2002).

Studies with α1(H101R) knock-in mice reveal that the sedative, amnesic and, in part, the anticonvulsant action of diazepam is mediated by α1-$GABA_A$ receptors (Rudolph et al., 1999). The effects of diazepam on electroencephalogram (EEG) patterns, such as REM-sleep suppression and reduction of slow-wave sleep, were largely unchanged after diazepam treatment in α1(H101R) knock-in mice, implying that EEG changes induced by diazepam are not mediated by α1-$GABA_A$ receptors (Tobler et al., 2001). Moreover, the sedative-hypnotic and anticonvulsant action of zolpidem is mediated by α1-$GABA_A$ receptors (Crestani et al., 2000). Zolpidem reduces REM sleep and increases non-REM sleep in both α1(H101R) knock-in and wildtype mice, implying that the effect of zolpidem on EEG sleep is not mediated by α1-$GABA_A$ receptors. However, the EEG power reduction in non-REM sleep is mediated by α1-$GABA_A$ receptors (Kopp et al., 2004a).

In α2(H101R) knock-in mice the anxiolytic and myorelaxant action of diazepam is absent at low diazepam doses (0.5 and 1 mg/kg) but the sedative and anticonvulsant action remains unaffected (Low et al., 2000; Morris et al., 2006). Thus α2-$GABA_A$ receptors

mediate the anxiolytic-like and myorelaxant actions of diazepam. Furthermore, the loss of the righting reflex by a combination of diazepam and low-dose alcohol is closely dependent on the activation of the α2-$GABA_A$ receptors (Tauber et al., 2003). In α2(H101R) knock-in mice, theta activity enhancement in REM sleep and waking by diazepam is reduced compared to wildtype mice. Furthermore the diazepam-induced suppression of delta activity in non-REM sleep is more pronounced in α2(H101R) knock-in mice, suggesting that α2-$GABA_A$ receptors are relevant for the modulation of rhythmic brain activities by benzodiazepines (Kopp et al., 2004b).

In α3(H126R) knock-in mice, the myorelaxant action of diazepam is partially removed (Crestani et al., 2001). The effects of diazepam on EEG sleep do not differ from wildtype, suggesting that α3-$GABA_A$ receptors are not critical for them (Kopp et al., 2003). This is also the case for α1(H101R)/ α3(H126R) double-mutant mice (Kopp et al., 2004a). In the thalamic reticular nucleus (nRt), the involvement of α3-$GABA_A$ receptors in thalamic giant depolarizing potentials (tGDPs) was analyzed with α3(H126R) knock-in mice. Benzodiazepine enhancement of tGDP amplitude and duration persisted in nRt neurons in α3(H126R) knock-in mice, indicating that the α3-$GABA_A$ receptors are not critical for tGDP generation, and that α5-$GABA_A$ receptors, which are transiently expressed in nRt neurons in early postnatal development, are probably involved (Pangratz-Fuehrer et al., 2007).

Also in α5(H105R) knock-in mice, the myorelaxant action of diazepam is partially removed (Crestani et al., 2001). α5(H105R) knock-in mice, which have a partial deficit of the α5-$GABA_A$ receptor, display selective changes in associative learning, memory performance and trace-fear conditioning (tone and foot shock separated by time) but not in delay-fear conditioning (tone and foot shock overlapping), and showed enhanced freezing compared to wildtype (Crestani et al., 2002). This indicates that α5-$GABA_A$ receptors are control elements of the temporal association of threat cues in trace-fear conditioning. Thus neuronal inhibition in the hippocampus mediated via α5-$GABA_A$ receptors is critical in controlling the regulation of the acquisition and expression of associative memory (Crestani et al., 2002; Yee et al., 2004). A downregulation of α5-$GABA_A$ receptors in the dentate gyrus is responsible for the manifestation of tolerance to the sedative action of diazepam. Thus, the chronic activation of α5-$GABA_A$ receptors is crucial for the normal

development of sedative tolerance to diazepam, which manifests itself in conjunction with α1-$GABA_A$ receptors (van Rijnsoever et al., 2004).

The knock-in mice were also used to determine the changes in transcript levels induced by acute diazepam administration. In wildtype animals, diazepam reduced the expression levels of the calcium/calmodulin-dependent protein kinase II, as well as those of brain-derived neurotrophic factor, MAP kinase phosphatase, transcription factor GIF, c-fos and nerve growth factor-induced gene-A. None of these transcripts were changed in the α1(H101R) knock-in mice after treatment with diazepam. Thus, the sedative action of diazepam is correlated with a selective downregulation of transcripts involved in the regulation of neuronal plasticity and neurotrophic responses. Most transcript changes are transient, except for the decrease of the CaMKIIα transcript. This long-term alteration may be involved in rebound phenomena and, under chronic treatment, in the development of tolerance and dependence (Huopaniemi et al., 2004).

Knock-in mice have provided the general result that $GABA_A$ receptors with different α subunits mediate distinct behavioral effects of diazepam, but the cellular correlates of the point mutation, as well as the neuronal circuits mediating this action, are still unknown. To address this question, genetically engineered mice carrying specific point mutations in a defined cell type and area needed to be created. Furthermore, with electrophysiological recordings it is potentially possible to assign a specific behavioral role to interneuronal circuits that signal through specific $GABA_A$ receptors. The selective drug sensitivity of the receptors in different knock-in mice therefore opens up the possibility of a detailed functional analysis. In this thesis we aim to characterize the functional role of different $GABA_A$ receptors in pyramidal cells and interneurons of the hippocampus using a combination of electrophysiological and immunohistochemical methods.

4.2. $GABA_A$ receptor expression and assembly in mice lacking individual $GABA_A$ receptor α subunits

Genetic ablation of individual $GABA_A$ receptor subunits revealed that the α subunit is required for receptor assembly. Therefore, mice lacking an α subunit gene provide insight into the function of specific $GABA_A$ receptor subtypes and allow us to address the question of whether compensatory changes in the expression of other $GABA_A$ receptor subunits or

other receptors or channels occur. The answers to these questions are important for interpreting the role of the receptor type and the plasticity of the inhibitory system.

So far, the α1, α3, α4, α5 and α6 subunits have been knocked out. α4, α5 and α6 subunits constitute a minor part of the $GABA_A$ receptor subtypes and are found predominantly in extrasynaptic sites in specific brain areas, as noted above. In the α5 knock-out mice, no upregulation of other α subunits was evident (Collinson et al., 2002), whereas in α6 knock-out mice the α1 subunit was also downregulated. In α4 knock-out mice, a decrease in the amount of tonic inhibition was observed, but no data about other α subunit expression exist (Chandra et al., 2006b). The fact that the knock-out of individual $GABA_A$ receptor subunits leads to secondary changes with respect to the formation of receptors, and compensatory upregulation, increases the complexity of the phenotypes and reflects the homeostatic regulation that prevents neuronal circuits from becoming hyper- or hypoactive.

Two lines of α1 knock-out mice ($\alpha1^{0/0}$) have been generated to examine the role of α1-$GABA_A$ receptors *in vivo* (Sur et al., 2001; Vicini et al., 2001). Although there is a loss of more than 50% of all $GABA_A$ receptors in $\alpha1^{0/0}$ mice, they are viable, and exhibit a mild phenotype (30% reduction in body weight and tremor when handling) (Kralic et al., 2002b). An upregulation of α2 and α3 subunits and a downregulation of β2/3 and γ2 subunits occurs in these mice, representing compensatory changes that suggest important network reorganization (Vicini et al., 2001; Kralic et al., 2002a; Kralic et al., 2006). The compensatory changes are region- and cell-type specific. The compensatory upregulation of $GABA_A$ receptors in $\alpha1^{0/0}$ mice does not show a change in regional or cellular distribution. For example, in Purkinje cells from cerebellum that express only the α1 subunit, a complete loss of GABAergic inhibitory currents is observed (Fritschy et al., 2006; Kralic et al., 2006). In brain regions where other α subunits are also expressed, an upregulation of these α subunits is observed. For example, the α3 subunit that is expressed in the molecular layer of the cerebellum, it is also increased in $\alpha1^{0/0}$ mice. Moreover, the extrasynaptic or synaptic localization of the receptors is not changed: receptors with an extracellular localization keep their extracellular localization, and synaptic receptors remain synaptic (Kralic et al., 2006). $\alpha1^{0/0}$ mice retain juvenile inhibitory synaptic currents until adulthood because mIPSCs have prolonged kinetics (Vicini et al., 2001; Goldstein et al., 2002; Bosman et al., 2005).

α1 knock-out mice lose the affinity for zolpidem (Kralic et al., 2002b) but are more sensitive to the sedative effects of diazepam, as determined by locomotor activity (Kralic et al., 2002b). This discrepancy reflects compensatory changes in the expression of $GABA_A$ receptors caused by the lack of the α1 subunit throughout development. Deletion of α1 subunit-containing receptors did not alter the anxiolytic, ataxic, anticonvulsant, or hypnotic effects of ethanol, or acute functional tolerance to ethanol, but did increase sensitivity to the locomotor-stimulating effects of ethanol (Kralic et al., 2003).

In conclusion, $\alpha1^{0/0}$ mice are a potent tool for studying several aspects of $GABA_A$ergic inhibition, such as: compensatory changes in $GABA_A$ receptor expression in different brain areas (Kralic et al., 2006; Ogris et al., 2006); changes in IPSCs (Goldstein et al., 2002; Bosman et al., 2005); pharmacological effects of benzodiazepines (Kralic et al., 2002a; Kralic et al., 2002b; Kralic et al., 2003); homeostatic inhibition (balance between phasic and tonic inhibition) (Ortinski et al., 2006); synaptogenesis (Fritschy et al., 2006); and developmental changes of inhibitory synaptic currents and quanta release (Vicini et al., 2001; Barberis et al., 2005; Bosman et al., 2005).

Using immunohistochemical and electrophysiological techniques, we used $\alpha1^{0/0}$ mice to study the compensatory changes that occur in defined hippocampal interneurons, and their functional consequences *in vivo.* Such a study allows us to learn about the plasticity of the inhibitory system at the cellular level and consequently provides information about defined neuronal circuits.

5. References

See page # 171

Aims

Using a combination of electrophysiological and immunohistochemical techniques, this thesis aims to investigate the functional significance of $GABA_A$ receptor diversity in the hippocampus, as well as the compensatory mechanisms by which the interneuronal GABAergic system overcomes the lack of an α1-$GABA_A$ receptor subtype. We focus mainly on the role of extrasynaptic receptors, which in recent years have been shown to be as important as synaptic receptors in $GABA_A$ergic inhibitory signaling. We address the following questions:

- Which $GABA_A$ receptors mediate phasic (fast and slow) and tonic forms of inhibition in CA1 pyramidal cells? (Publication 1)
- Can we selectively stimulate a type of $GABA_A$ receptor? (Publication 1)
- Do slow events and tonic currents share the same pool of extrasynaptic receptors? (Publication 1)
- Does tonic inhibition exists when the GABA uptake machinery is intact in pyramidal cells and interneurons? (Publications 1 and 2)
- Which $GABA_A$ receptors mediate tonic inhibition in CA1 interneurons? (Publication 2)
- What determines the differences in tonic conductance in interneurons? Is it an intrinsic property of the cells? (Publication 2)
- How can we use immunofluorescence techniques to visualize synaptic $GABA_A$ receptors in identified interneurons? (Publication 3)
- Which changes occur in $GABA_A$ receptor expression and location in α1 knock-out mice ($\alpha1^{0/0}$)? (Publication 4)
- What are the functional consequences of the lack of the α1 subunit in interneurons? (Publication 4)
- Are the compensatory changes in $\alpha1^{0/0}$ mice sufficient to protect against a pathological challenge such as epilepsy? (Publication 4)

1. Specific subtypes of $GABA_A$ receptors mediate phasic and tonic forms of inhibition in hippocampal pyramidal neurons (Publication 1)

We performed whole-cell voltage-clamp recordings in pyramidal cells from wildtype, single (α1, α2, α3), double (α12) and triple (α123) knock-in mice (described in the Introduction, section 4.1) to determine which α-containing $GABA_A$ receptors are involved in: 1) Evoked inhibitory postsynaptic currents (eIPSCs) with stimulation proximal or distal to the soma; 2) Spontaneous fast and slow inhibitory postsynaptic currents (sIPSC); and 3) Tonic inhibition under intact uptake machinery. To assess whether tonic inhibition and slow sIPSCs share the same pool of α-containing $GABA_A$ receptors, we investigated the effects of high and low doses of picrotoxin (PIC) on tonic currents, and fast and slow sIPSCs from wildtype mice. The remaining experiments described in publication 1 were performed by my colleague G. Prenosil.

2. Interneurons in the hippocampal CA1 area exhibit region-specific tonic inhibition mostly mediated by α1-$GABA_A$ receptors (Publication 2)

We performed whole-cell voltage-clamp recordings in stratum oriens, stratum radiatum and stratum lacunosum-moleculare interneurons from wildtype, single (α1, α2, α3, α5), double (α12, α23) and triple (α123) knock-in mice (described in the Introduction, section 4.1), to analyze the involvement of α-containing $GABA_A$ receptors in tonic inhibition. To analyze whether differences in tonic conductance are an intrinsic property of interneurons, we recorded the different spiking patterns in current clamp.

3. Immunofluorescence in brain sections: simultaneous detection of presynaptic and postsynaptic proteins in identified neurons (Publication 3, option C)

The immunofluorescent visualization of the expression of different $GABA_A$ receptors in identified interneurons was limited by the fact that the strong tissue fixation required for optimal detection of most intracellular markers (such as calcium-binding proteins and

neuromodulators) can mask several postsynaptic epitopes (such as $GABA_A$ receptors and gephyrin). We therefore investigated several types of tissue preparation and fixation in order to simultaneously achieve the optimal preservation of cell morphology and the detection of soluble cell markers and synaptic proteins. We succeed in develope a novel protocol overcoming the mentioned limitations.

4. Reorganization of GABAergic circuits maintains $GABA_A$ receptor-mediated transmission onto CA1 interneurons in α1 subunit-null mice (Publication 4)

By applying the newly established immunofluorescence protocol (described in Publication 3), and whole-cell voltage-clamp recordings from interneurons in wildtype and α1 knock-out mice ($\alpha1^{0/0}$), we studied the compensatory changes in $GABA_A$ receptor expression and localization in five major populations of interneurons. We then investigated the sIPSCs, miniIPSCs (mIPSCs) and tonic $GABA_A$ergic currents from $\alpha1^{0/0}$ mice and tested the susceptibility of $\alpha1^{0/0}$ mice against kainic acid-induced seizures. The patch clamp recordings in this study were performed by G. Prenosil and V. Duveau performed the kainic acid injection analysis.

Results

1. Specific subtypes of $GABA_A$ receptors mediate phasic and tonic forms of inhibition in hippocampal pyramidal neurons

George A. Prenosil[1], Edith M. Schneider Gasser[1], Uwe Rudolph, Ruth Keist, Jean-Marc Fritschy and Kaspar E. Vogt

[1]These authors contributed equally to the study

Published in: Journal of Neurophysiology 96: 846-857, 2006.

Abstract

The main inhibitory neurotransmitter in the mammalian brain, γ-aminobutyric acid (GABA), mediates multiple forms of inhibitory signals, such as fast and slow inhibitory postsynaptic currents and tonic inhibition, by activating a diverse family of ionotropic $GABA_A$ receptors. Here, we have investigated whether distinct $GABA_A$ receptor subtypes mediate these various forms of inhibition using as approach mice carrying a point mutation in the α-subunit rendering individual $GABA_A$ receptor subtypes insensitive to diazepam without altering their GABA sensitivity and expression of receptors. Whole-cell patch-clamp recordings were performed in hippocampal pyramidal cells from single, double, and triple mutant mice. Comparing diazepam effects in knock-in and wildtype mice allowed determining the contribution of α1, α2, α3, and α5 subunit containing $GABA_A$ receptors to phasic and tonic forms of inhibition. Fast phasic currents were mediated by synaptic α2-$GABA_A$ receptors on the soma and by synaptic α1-$GABA_A$ receptors on the dendrites. No contribution of α3- or α5-$GABA_A$ receptors was detectable. Slow phasic currents were produced by both synaptic and perisynaptic $GABA_A$ receptors, judged by their strong sensitivity to blockade of GABA reuptake. In the CA1 area, but not in the subiculum, perisynaptic α5-$GABA_A$ receptors contributed to slow phasic currents. In the CA1 area the diazepam-sensitive component of tonic inhibition also involved activation of α5-$GABA_A$ receptors and slow phasic and tonic signals shared overlapping pools of receptors. These results demonstrate that the major forms of inhibitory neurotransmission in hippocampal pyramidal cells are mediated by distinct $GABA_A$ receptors subtypes.

Introduction

In the mammalian central nervous system, α-aminobutyric acid (GABA) is the main inhibitory neurotransmitter, activating $GABA_A$ receptors on target neurons either in a phasic or a tonic fashion (Farrant and Nusser 2005; Mody and Pearce 2004). $GABA_A$ receptors are composed of five subunits, most frequently two α, two β, and one γ subunit. Different subunit isoforms (α1-6, β1-4, γ1-3, δ, ε, π, θ) give rise to a considerable diversity of $GABA_A$ receptors (Barnard et al. 1998; Mohler et al. 2002; Sieghart and Sperk 2002) that are differentially expressed in the brain and localized in different cell types and subcellular areas (Fritschy and Mohler 1995; McKernan and Whiting 1996; Pirker et al. 2000). Although much is known about the distribution and subcellular location of major $GABA_A$ receptor subtypes, the functional significance of this diversity is less well understood, chiefly due to a lack of pharmacological tools that distinguish between the different subtypes. The presence of a γ2 or γ3 subunit is required for the formation of a benzodiazepine-binding site (Knoflach et al. 1991; Pritchett et al. 1989). Additionally, the α subunit variant determines activation and de-activation kinetics of $GABA_A$ receptors (Banks and Pearce 2000; Bosman et al. 2002; Hutcheon et al. 2000; Vicini et al. 2001) and their affinity for classical benzodiazepines (Scholze et al. 1996; Smith et al. 2001) such as diazepam (diazepam). Mutation of a conserved histidine residue (His 101 in the α1 subunit) into an arginine renders the corresponding $GABA_A$ receptor diazepam-insensitive (Benson et al. 1998; Rudolph et al. 1999; Wieland et al. 1992). Using gene targeting to introduce this point-mutation in various α subunit variants *in vivo*, it has been shown that different $GABA_A$ receptor subtypes mediate distinct effects of diazepam (Rudolph and Mohler 2004). In particular, its sedative properties are mediated by α1-$GABA_A$ receptors (McKernan et al. 2000; Rudolph et al. 1999) and its anxiolytic effects by α2-$GABA_A$ receptors (Low et al. 2000). It can therefore be assumed that specific neuronal circuits use distinct $GABA_A$ receptor subtypes differing in α subunit variant.

Two major modes of $GABA_A$ receptor-mediated inhibitory transmission can be observed in mammalian CNS: phasic inhibition mediated by synaptic receptors and tonic inhibition mediated by extrasynaptic receptors (Farrant and Nusser 2005; Mody and Pearce 2004). In the hippocampal formation, phasic inhibitory postsynaptic currents (IPSCs) can further be subdivided into $GABA_{A,\ fast}$ and $GABA_{A,\ slow}$ IPSCs based on kinetics and amplitude (Pearce 1993). $GABA_{A,\ fast}$ IPSCs are mediated mostly by somatic and proximal dendritic

synapses (Freund and Buzsaki 1996), whereas $GABA_{A, slow}$ IPSCs are thought to originate at distal dendritic sites (Banks et al. 1998). $GABA_{A, slow}$ IPSCs exhibit on average a larger charge transfer than $GABA_{A, fast}$ IPSCs; their prolonged time course and their sensitivity to GABA reuptake inhibitors (Banks et al. 2000) suggested that they are activated by GABA spillover.

The subunit composition and function of tonically activated $GABA_A$ receptors varies across brain regions and cell types. In cerebellum, dentate gyrus, neocortex and thalamic relay nuclei, tonic inhibition is mediated by diazepam-insensitive $GABA_A$ receptors containing α6 or α4 subunits together with the δ subunit (Brickley et al. 1996; Nusser and Mody 2002; Porcello et al. 2003; Stell et al. 2003; Sun et al. 2004; Cope et al., 2005; Drasbek and Jensen, 2005; Mtchedlishvili and Kapur, 2006). In the CA1 area, tonic inhibition is mediated by diazepam-sensitive $GABA_A$ receptors in interneurons and, to lesser extent pyramidal cells, when ambient GABA concentration is increased (Scimemi et al. 2005; Semyanov et al. 2003). At low GABA concentrations activation of $GABA_A$ receptors containing the α4 subunit was found to dominate (Scimemi et al. 2005). So far, however, no single $GABA_A$ receptor subtype has been found to have an exclusive synaptic or extrasynaptic location, but several lines of evidence suggest that specific $GABA_A$ receptors selectively participate in different forms of inhibition. Unraveling this selectivity would help understanding how distinct behaviors and diazepam effects are linked with the diverse forms of GABAergic inhibition.

The aim of the present work was to investigate whether diazepam-sensitive $GABA_A$ receptors differing in α subunit composition mediate distinct modes of GABAergic inhibition in the hippocampal formation. Whole-cell patch-clamp recordings of CA1 and subicular pyramidal cells were performed on acute slices from knock-in mice carrying a histidine-to-arginine point-mutation in either the α1, α2 or α3 subunit gene to render the respective $GABA_A$ receptor diazepam-insensitive. Using this approach in single (α1, α2, α3), double (α12), and triple (α123) mutant mice to pharmacologically isolate $GABA_A$ receptors containing different α subunits, we investigated the contribution of identified diazepam-sensitive $GABA_A$ receptor subtypes to evoked and spontaneous IPSCs and to tonic inhibition in the hippocampus. A particular focus was put on characterization of $GABA_{A, slow}$ IPSCs, as they share elements of both phasic and tonic type of GABAergic inhibition.

Materials and Methods

Generation of mutant mice

Experiments were performed in 129/SvJ knock-in mice carrying diazepam-insensitive $GABA_A$ receptor subtypes obtained by a histidine-to-arginine point-mutation in the α1, α2, or α3 subunit gene [α1(H101R), α2(H101R) and α3(H126R)] (Low et al. 2000; Rudolph et al. 1999) introduced into the mouse genome by homologous recombination in embryonic stem cells. Mice carrying a point mutation in both α1 and α2 subunits (α12) were obtained by crossing double heterozygous mice born from homozygous α1(H101R) and α2(H101R) intercrosses. Homozygous α1(H101R)/α2(H101R) offspring were identified by PCR analysis from tail biopsy. Likewise, triple mutants, carrying point-mutated α1, α2 and α3 subunits (α123) were generated by crossing double homozygous α1(H101R)/α2(H101R) and α1(H101R)/α3(H126R) mice, which yielded offspring homozygous for α1(H101R) but heterozygous for α2(H101R) and α3(H126R) subunits. These were crossed again with each other to obtain mice homozygous for all three point-mutations, as confirmed by PCR analysis. All experiments were approved by the cantonal Veterinary Office of Zurich and were performed in accordance with the European Community Council Directive (86/609/EEC).

Immunohistochemistry

Mice (P21) were anaesthetized with sodium pentobarbital (Nembutal, 50 mg/kg i.p., Abbot Laboratories, Chicago, IL) and were perfused transcardially with 4% paraformaldehyde dissolved in 0.15 M sodium phosphate buffer containing 15% saturated picric acid solution. The brain was extracted, post-fixed for 4 h in the same solution, incubated overnight in sodium citrate buffer (pH 4.5), and boiled for 60 sec in a microwave oven for antigen retrieval (Fritschy et al. 1998). They were then cryoprotected with 30% sucrose in phosphate buffered saline, frozen with dry-ice and cut in 40 μm parasagittal sections using a freezing microtome. Free-floating sections were processed for immunoperoxidase staining using antibodies against the α1, α2, α3, and α5 subunit, as described (Fritschy et al. 1998). For antibody characterization see Fritschy and Mohler (1995).

Slice preparation

Mice from both sexes (P18-24) were anaesthetized with inhaled isoflurane and decapitated. The brain was quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF, composition in mM: NaCl 125, $NaHCO_3$ 26, NaH_2PO_4 1.25, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2.5, glucose 11, oxygenated with 95% O_2 / 5% CO_2). The brain was affixed to a vibratome stage (Microm HM 650 V, Microm International AG, Volketswil, Switzerland) with cyanoacrylate and kept in the ice-cold ACSF for slicing. Parasagittal 300-350 μm thick hippocampal slices were prepared and incubated at 33°C for 20 min, prior to being stored at room temperature (25°C) in oxygenated ACSF. For the recording of $GABA_{A,\ slow}$ IPSCs, mice (P24-30) were anaesthetized with Nembutal and were perfused transcardially with 50 ml ice-cold sucrose-ACSF (composition in mM: NaCl 87, $NaHCO_3$ 26, NaH_2PO_4 1.25, KCl 2.5, $MgCl_2$ 9, $CaCl_2$ 0.5, sucrose 75, glucose 25). Parasagittal slices (310 μm thick) were cut in sucrose-ACSF, transferred to normal ACSF, and incubated and stored as above.

Electrophysiological recordings and data analysis

Slices were visualized with a CCD camera (PCO Vx45; Till Photonics, Germany) mounted on an upright microscope (BX51WI; Olympus, Switzerland), equipped with a long working distance water-immersion objective (Xlumplan FI 20X, 0.95 numerical aperture), a fourfold magnification changer, Nomarski-type differential interference contrast and infrared illumination. Patch electrodes were pulled from borosilicate glass (GC150TC; Clark Instruments, UK) and had an open tip resistance of 3-4 MΩ when filled with the internal solution. 2 mM kynurenic acid was added to the external ACSF solution to block excitatory synaptic transmission. Recordings were made using a Multiclamp 700A patch-clamp amplifier (Axon Instruments, Inc. USA), filtered at 4 kHz, digitized at 20 kHz, stored and analyzed using IGOR Pro software (Wave Metrics, Lake Oswego, USA). Access resistance was monitored for all experiments and they were not included in further analysis if it changed by more than 20% during the recording.

Evoked IPSCs

Whole-cell voltage-clamp recordings from CA1 pyramidal cells of wildtype (WT), α1, α2, α3, α12 and α123 mutant mice were made at room temperature (25°C) with continuous superfusion (1-2 ml/min) of ACSF. External stimulation (0.1-10 μA) was delivered every

10 sec with a constant current stimulus isolator (WPI, Florida, USA) through a bipolar, custom-made electrode from polytetrafluoroethylene-insulated platinum-iridium wire of 50 μM in diameter (Advent, UK). Evoked IPSCs (eIPSC) were recorded at a holding potential of 0 mV with a low chloride containing internal solution (composition in mM: CsGlu 130, EGTA 1, HEPES 10, MgATP 5, NaGTP 0.5, NaCl 5, pH 7.3, 295 mOsm). Access resistance was around 10-12 MΩ.

To assess the contribution of different $GABA_A$ receptor subtypes to eIPSCs in slices from WT and knock-in mice, diazepam (1 μM, dissolved in DMSO) was bath-applied after 15 minutes of baseline recordings and eIPSCs were recorded for another 15-30 minutes. The average eIPSC amplitude after application of diazepam was normalized to the peak amplitude of the average baseline eIPSC. No significant difference in the diazepam effect was observed between mono- and bi-exponential fittings, and therefore we chose the mono-exponential method. The increase in amplitude and τ after diazepam application in each line of mutant mice was compared to wildtype mice using one-way ANOVA followed by Bonferroni's post-hoc multiple comparisons tests (SPSS 11.5; Lead Technology, USA). In all experiments "n" refers to the number of cells recorded. In average 1 to 2 cells were recorded from one animal.

Spontaneous $GABA_{A, fast}$ and $GABA_{A, slow}$ IPSCs

We recorded spontaneous GABAergic events to reliably distinguish between $GABA_{A, slow}$ and $GABA_{A, fast}$ and to facilitate the comparison of results from two hippocampal regions. Whole-cell voltage-clamp recordings of spontaneous IPSCs (sIPSC) from CA1 and subiculum pyramidal cells from wildtype and α123 mutant mice were obtained at room temperature with a holding potential of –60 mV and a high chloride containing internal solution (composition in mM: CsCl 100, $MgCl_2$ 2, EGTA 1, ATP 2, GTP 0.3, HEPES 40, pH 7,2, 300 mOsm). Experiments with the GABA reuptake inhibitor NO711 (2 μM, dissolved in DMSO) were performed in the presence of the $GABA_B$ receptor antagonist CGP 55845 (1 μM, dissolved in DMSO). Continuous recordings started after the holding current had stabilized. Spontaneous events were recorded for a 10-15 min baseline period and for the same amount of time in the presence of each drug tested. Banks *et al*. (2002) reported an increase in frequency of $GABA_{A, slow}$ sIPSCs with age in rats, while other parameters such as amplitude and kinetics remained unchanged. We checked for the age dependency of $GABA_{A, slow}$ sIPSCs in wildtype mice by recording from 34 animals ranging

from P15 to P35. As in rats, the frequency of $GABA_{A,\ slow}$ sIPSCs was higher in older animals. However, we observed a steep increase around P19 (results not shown), whereas in rats the mean frequency of $GABA_{A,\ slow}$ sIPSCs was reported to increase gradually with age. Based on this result, all subsequent sIPSC recordings were performed in mice between P20 and P25.

Spontaneous events were detected offline automatically with the 'Mini Analysis' software (Synaptosoft, USA) with the detection threshold being set five times higher than the RMS level of baseline noise. All detected events were counted for analysis of frequency. For analysis of kinetics and amplitude only currents without subsequent contaminating events in the decaying phase were considered. Spontaneous events having an onset-to-peak rise time greater than 5 ms were classified as $GABA_{A,\ slow}$ sIPSCs, remaining events were classified as $GABA_{A,\ fast}$ sIPSCs (Banks et al. 2000). Amplitude, rise time and decay time constants for single $GABA_{A,\ slow}$ sIPSCs were calculated by the 'Mini Analysis' software. Kinetics of $GABA_{A,\ fast}$ sIPSCs was also calculated individually, whereas their amplitude was determined from an average trace for each recorded cell. This minimized contamination by noise as, in general, the signal-to-noise ratio was smaller for $GABA_{A,\ fast}$ than for $GABA_{A,\ slow}$ sIPSCs. The effects of diazepam and NO711 on sIPSCs were calculated from the average value of pooled sIPSCs from single experiments before and after drug application. Statistical significance was determined by paired Student's t-tests.

Tonic inhibition recordings

Tonic inhibition was determined by the change in holding current after application of the $GABA_A$ receptor antagonist picrotoxin (2 or 100 μM dissolved in DMSO). Sensitivity to diazepam (1 μM) or L-655,708 (5 μM) was measured by first applying the drug to the bath solution and then assessing the effect of picrotoxin. Whole-cell recordings from CA1 pyramidal cells in wildtype and α123 mutant mice were made at a holding potential of -60 mV with a high chloride containing internal solution. $GABA_B$ receptors were blocked with the antagonist CGP 55845 (1 μM). Recordings were analyzed with the 'Mini Analysis' software with the detection threshold set five times higher than the level of baseline noise. The holding current was measured during 10 ms segments preceding spontaneous events, to avoid contamination with phasic currents. The average holding current was calculated in recordings taken after chloride equilibration (baseline), ~200 sec after diazepam application, ~120 - 150 seconds after picrotoxin application (t1), and ~240 – 300 seconds

after picrotoxin application (t2). For each experiment, changes in holding current after drug application were statistically compared to baseline with paired Student's t-test. Differences between genotypes were analyzed with one-way ANOVA and Bonferroni's post-hoc multiple comparisons tests.

Drugs

Chemicals were purchased from Sigma/Fluka (Switzerland) or Tocris Inc. (ANAWA Inc, Switzerland), diazepam was provided by Hoffmann-La Roche Ltd (Switzerland). We tested DMSO alone at the appropriate concentrations on IPSCs to rule out direct effects when it was used as a solvent.

Results

$GABA_A$ receptors subtypes expressed in CA1 area

The distribution of the four α subunit variants (α1, α2, α3, α5) contributing to diazepam-sensitive $GABA_A$ receptors was analyzed in the hippocampal formation by immunoperoxidase staining in P21 wildtype mice, revealing clear-cut differences in staining intensity and regional distribution (Fig.1). Although staining intensity cannot be compared across antibodies, comparisons can be made relative to other brain regions. Thus, the α2 and α5 subunit exhibited the strongest immunoreactivity in the hippocampal formation, with α2 being more intense in the dentate gyrus than in CA1, whereas the α5 subunit was strongest in CA1, especially in the pyramidal cell layer. Staining for both subunits was homogeneous across dendritic and cell body layers of the hippocampal formation, suggesting the presence of the corresponding receptors on the soma and dendrites of pyramidal cells. As reported previously (Brünig et al. 2002), the α1 subunit antibody strongly labelled a population of interneurons throughout the hippocampal formation and produced only a moderate staining in the dendritic layers of CA1, CA3, and the dentate gyrus, while the cell body layers appeared almost devoid of staining. The α3 subunit was expressed at low levels in CA1, and was present in a few isolated interneurons, mainly found in the stratum oriens and in the hilus. The boundary between CA1 and subiculum was particularly evident for the α5 subunit, which is almost not detectable in the latter region.

Examination of sections from P21 and adult single and triple knock-in mice revealed no difference in regional distribution and relative staining intensity for the four subunits analyzed (not shown), as reported previously for the amygdala (Marowsky et al. 2004) and the cerebral cortex (Fagiolini et al. 2004).

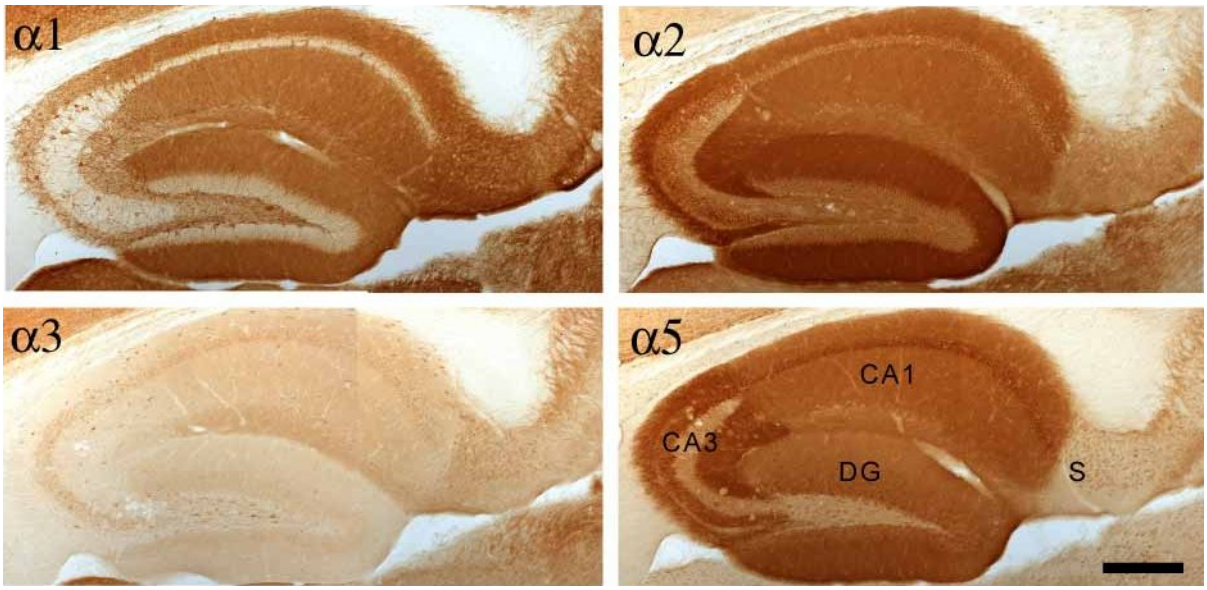


Figure 1. Differential distribution of α subunit variants contributing to diazepam-sensitive $GABA_A$ receptors in the hippocampal formation. Parasagittal sections from P21 wildtype mice were processed for immunoperoxidase staining with subunit-specific antibodies. The α1, α2, and α5 subunit have overlapping, but distinct distribution in CA1, CA3 and the dentate gyrus (DG). In contrast, staining for the α3 subunit is much weaker in the hippocampus than deep cortical layers (upper right corner) or superior colliculus (lower right corner). Both the α1 and α3 subunit label interneurons in various subfields. In subiculum (S), staining for the α1 and α2 subunit is prominent, whereas α3 is weakly stained and α5 absent, marking a sharp boundary with the CA1 subfield. Scale bar, 300 μm.

Differential contribution of α1- and α2-$GABA_A$ receptors to evoked synaptic inhibition in CA1 pyramidal cells

To assess the major $GABA_A$ receptor subtypes mediating phasic inhibition, the effects of diazepam on eIPSCs were compared in wildtype mice and five lines of mutant mice carrying diazepam-insensitive $GABA_A$ receptors (α1, α2, α3, α12, and α123 knock-in mice). Current pulses were delivered every 10 seconds through an extracellular stimulus electrode and their intensity adjusted to generate eIPSCs of similar amplitude in all

experiments (Table 1). There was no difference in rise time and decay time constants of eIPSCs in the different genotypes investigated (Table 1).

Bath-application of diazepam (1 μM) invariably increased the amplitude of eIPSCs in wildtype mice (49 ± 5 %, n = 14) (Fig. 2A, left trace) indicating that $GABA_A$ receptors are not saturated in our recording conditions. The normalized effect of diazepam on eIPSC peak amplitude (46 ± 8%, n = 13) and decay time constant (29 ± 3%, n = 13) was not different in α3 knock-in mice compared to wildtype (Figure 2B, C), whereas in α12 and α123 knock-in mice it was abolished (amplitude: 6 ± 5 %, n = 7 and 6 ± 2%, n = 7) (Figure 2A, right trace). In contrast, diazepam produced a significant increase in peak amplitude (α1: 33 ± 5%, n = 15 and α2: 27 ± 8%, n = 10) and decay time in recordings from α1 and α2 knock-in mice (Figure 2B, C). Therefore, no contribution of either α3- or α5-$GABA_A$ receptors to evoked phasic inhibition could be resolved in these mice, whereas both α1- and α2-$GABA_A$ receptors mediate the bulk of eIPSCs in CA1 pyramidal cells.

To determine whether these two receptor subtypes are segregated between the soma and dendrites, the stimulus electrode was placed either in the stratum pyramidale (proximal stimulation) or at the border between stratum radiatum and stratum lacunosum-moleculare (distal stimulation) (Fig. 3). With proximal stimulation, diazepam application produced an increase in peak eIPSC amplitude in α1 knock-in mice similar to wildtype (50 ± 10%, n = 7; $P < 0.02$), whereas in α2 knock-in mice the increase was much smaller (8 ± 4%, n = 5; $P < 0.002$). The opposite effect was observed in recordings with distal stimulation (Figure 3): in α2 knock-in mice, the increase in amplitude after diazepam application (46 ± 16%, n = 5, $P < 0.002$) was similar to wildtype mice whereas in α1 knock-in mice it was only 20 ± 5 % (n = 8, $P < 0.02$). Therefore, α2-$GABA_A$ receptors preferentially mediate phasic synaptic inhibition on the soma and α1-$GABA_A$ receptor on the dendrites of CA1 pyramidal cells, in line with the subcellular distribution of these subunits (Fig. 1). The fact that the activation of distinct $GABA_A$ receptor subtypes can be resolved in these knock-in mice makes them a suitable tool for further investigation of GABAergic transmission in the hippocampal formation.

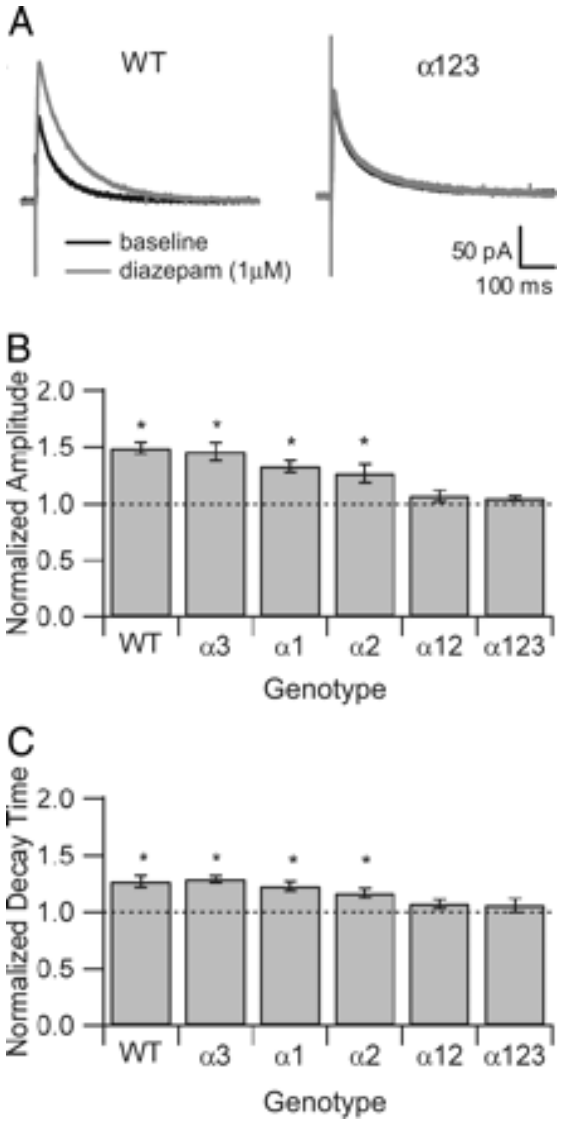


Figure 2. Effect of diazepam (1 μM) on evoked IPSCs in WT and knock-in mice. A: samples of averaged eIPSCs obtained before (baseline) and after diazepam application in WT and in α123 knock-in mice; note the lack of contribution of α5-$GABA_A$ receptors. B and C: normalized averaged diazepam effect on peak amplitude (B) and decay time constant (C) in the different genotypes tested: WT, α1, α2, and α3 knock-in mice showed a significant increase in amplitude and decay time after application of diazepam (* $P < 0.05$, paired Student's t-test). ANOVA tests indicated a significant difference between genotypes ($P < 0.001$, $n = 66$) for both amplitude and decay time after diazepam. Post-hoc analysis with Bonferroni multiple comparisons tests revealed significantly larger responses in WT and α3 knock-in mice than in α12 and α123 knock-in mice ($P \leq 0.001$ for amplitude, and $P \leq 0.026$ for decay time). Numbers of experiments per genotype are shown in table 1.

TABLE 1. ***Baseline properties of eIPSCs recorded from CA1 pyramidal cells in wildtype (WT) and knock-in mice***

Genotype	*n*	Amplitude, pA	Rise time, ms	Decay time constant, ms
WT	14	120 ± 10	4.2 ± 0.5	64.5 ± 6
α3	13	110 ± 9	5.7 ± 0.9	64.7 ± 7.6
α1	15	109 ± 12	5.5 ± 0.7	67.9 ± 5.3
α2	10	125 ± 10	5.4 ± 0.6	74.0 ± 6.3
α12	7	124 ± 28	3.7 ± 1.3	59.0 ± 8.2
α123	7	107 ± 21	5.3 ± 1.0	63.1 ± 10.7

Values are given as mean ± SE. eIPSC, evoked inhibitory postsynaptic current; WT, wildtype.

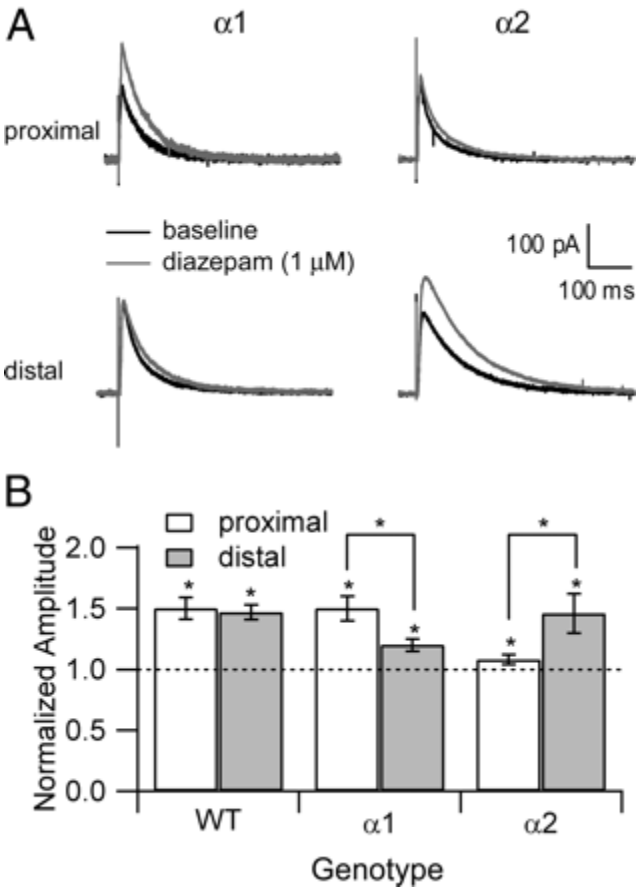


Figure 3. Effect of diazepam (1 μM) on eIPSCs reveals a genotype effect according to the area of stimulation. *A*: examples of averaged eIPSCs obtained before (baseline) and after diazepam application in α1 (*left*) and α2 (*right*) knock-in mice according to the area of stimulation: either proximal (*top*) or distal (*bottom*) to the cell soma. *B*: in WT mice, diazepam-induced amplitude increase of eIPSCs was similar in proximal and distal evoked responses (n = 14, not significant). In α1 knock-in mice, the diazepam-induced amplitude increase of eIPSCs was similar to WT in proximally evoked responses, but significantly reduced in distally evoked responses ($P < 0.02$, n = 15, unpaired Student's t-test, equal variances assumed). In α2 knock-in mice, diazepam application affected proximally evoked responses significantly more than distally evoked responses ($P < 0.002$, n = 10, unpaired Student's t-test, equal variances assumed). *Significant changes from baseline ($P < 0.05$, paired Student's t-test).

Distinct $GABA_A$ receptor subtypes mediate fast and slow sIPSCs

$GABA_{A,\ fast}$ and $GABA_{A,\ slow}$ IPSCs represent two distinct modes of phasic synaptic inhibition in CA1 pyramidal cells, best distinguished in recordings of spontaneous events. $GABA_{A,\ slow}$ IPSCs have been suggested to involve a mixture of synaptic and perisynaptic or extrasynaptic receptors and to be mediated by a specialized, yet not identified, type of interneuron (Banks et al. 2000). Although α5-$GABA_A$ receptors do not appear to be much involved in eIPSCs (Fig. 2), a contribution to $GABA_{A,\ slow}$ IPSCs is conceivable. Therefore, to determine whether different $GABA_A$ receptors mediate $GABA_{A,\ fast}$ and $GABA_{A,\ slow}$ sIPSCs, the effects of diazepam were assessed in α123 knock-in and wildtype mice, using continuous whole-cell voltage-clamp recordings from CA1 and subicular pyramidal cells.

Baseline properties of sIPSCs (peak amplitude, rise time and decay time constant) did not differ significantly between genotypes and hippocampal regions (CA1, subiculum) (Tables 2 and 3). Examples of recordings from a CA1 pyramidal cell are shown in Fig. 4A, D.

sIPSCs were analyzed and subdivided into $GABA_{A,\ slow}$ (Fig. 4B, E) and $GABA_{A,\ fast}$ sIPSCs (Fig. 4C, F), as described in the methods section.

After baseline recording, diazepam (1 μM) was bath-applied and recording was continued until enough $GABA_{A,\ slow}$ sIPSCs were detected for statistical analysis. diazepam significantly increased the mean amplitude of $GABA_{A,\ slow}$ sIPSCs in α123 knock-in mice from -83.5 ± 6.1 pA to -111.2 ± 9.8 pA ($P < 0.01$, $n = 9$) and in wildtype mice from -117.0 ± 17.4 pA to -229.3 ± 46.7 pA ($P < 0.05$, $n = 7$). In addition, a significant increase in decay time constant was observed in wildtype mice, from 40.7 ± 4.0 ms to 63.0 ± 9.6 ms after diazepam application ($P < 0.05$, $n = 7$), whereas the increase in decay time in α123 knock-in mice just failed to reach significance. The rise time of $GABA_{A,\ slow}$ sIPSCs did not change significantly after diazepam application in either genotype.

The effect of diazepam in α123 knock-in mice indicates that α5-$GABA_A$ receptors participate in $GABA_{A,\ slow}$ sIPSCs. However, the increase in $GABA_{A,\ slow}$ sIPSCs in CA1 pyramidal cells was significantly larger in wildtype than in α123 knock-in mice (two-tailed, unpaired Student's *t*-test, $n_{WT} = 7$, $n_{\alpha123} = 8$), suggesting that α1- and/or α2-$GABA_A$ receptors also contribute to $GABA_{A,\ slow}$ sIPSCs. In contrast, $GABA_{A,\ fast}$ sIPSCs were not affected by diazepam in α123 knock-in mice, unlike in wildtype (Fig. 4N, O). These findings are consistent with the observations made on eIPSCs and suggest that the contribution of α5-$GABA_A$ receptors to phasic inhibition is restricted to $GABA_{A,\ slow}$ IPSCs.

TABLE 2. ***Baseline properties of $GABA_A$, slow sIPSCs***

Genotype and Region	n	Amplitude, pA	Rise time, ms	Decay time constant, ms
WT, CA1	7*	-117.0 ± 17.4	20.6 ± 0.8	40.7 ± 4.0
α123, CA1	9	-83.5 ± 6.1	18.2 ± 1.3	31.7 ± 3.3
WT, Sub	6	-72.7 ± 12.0	11.6 ± 0.7	22.0 ± 0.7
α123, Sub	7	-123.2 ± 22.8	17.1 ± 1.6	31.6 ± 3.1

Values are means ± SE. * In 2 out of 9 cells the number of $GABA_{A,\ slow}$ sIPSCs was too low for statistical analysis. Sub, subiculum. For other abbreviations see Table 1.

TABLE 3. ***Baseline properties $GABA_A$, fast sIPSCs***

Genotype and Region	n	Amplitude, pA	Rise time, ms	Decay time constant, ms
WT, CA1	9	-44.2 ± 3.7	0.48 ± 0.04	16.3 ± 2.7
α123, CA1	6	-44.5 ± 2.3	0.51 ± 0.06	18.7 ± 9.5
WT, Sub	6	-40.6 ± 3.3	0.45 ± 0.03	7.7 ± 0.8
α123, Sub	7	-62.8 ± 6.9	0.52 ± 0.07	11.9± 4.4

Values are means ± SE. For abbreviations, see Tables 1 and 2.

As shown in Fig. 4, α5 subunit immunoreactivity is almost undetectable in subiculum, just adjacent to the CA1 area, allowing to characterize $GABA_{A,\ slow}$ sIPSCs in neurons lacking α5-$GABA_A$ receptors and to validate the findings obtained in α123 knock-in mice. Experiments were performed applying the same protocol used for the CA1 area (Fig. 4G, K). $GABA_{A,\ slow}$ sIPSCs were observed in baseline recordings of subicular pyramidal cells, albeit at a lower frequency than in CA1. ($f_{Subiculum} = 0.039 \pm 0.005\ s^{-1}$; $f_{CA1} = 0.099 \pm 0.012\ s^{-1}$). Bath-application of diazepam (1 μM) increased the amplitude and decay time constant of both $GABA_{A,\ slow}$ and $GABA_{A,\ fast}$ sIPSCs, in cells from wildtype mice (Fig. 4H, J, N, and O), while in α123 knock-in mice these values remained unchanged (Fig. 4L, M, N, and O). In addition, application of the inverse agonist at the benzodiazepine binding site L-655,708 (5 μM) only affected the amplitude of $GABA_{A,\ slow}$ in wildtype CA1 pyramidal cells (–74.1 ± 12.4 pA to –58.1 ± 9.5 pA, n=7, $P<0.05$), but not in subicular pyramidal cells (n = 5). Amplitude and kinetics of $GABA_{A,\ fast}$ sIPSCs in CA1 and subiculum (n=7 and n = 5, respectively) remained unchanged. L-655,708 exhibits at least 50 times higher affinity for α5-$GABA_A$ receptors over $GABA_A$ receptors containing other α subunits (Quirk *et al.* 1996). These results further confirmed that α5-$GABA_A$ receptors are absent in the subiculum and thus are not required for the generation of $GABA_{A,\ slow}$ and that these receptors do not contribute to $GABA_{A,\ fast}$ sIPSCs.

Due to their slow time-course and sensitivity to GABA reuptake inhibitors, $GABA_{A,\ slow}$ IPSCs have been proposed to involve, at least in part, extrasynaptic receptors activated by GABA spillover (Banks et al. 2000). To compare the subunit profile of synaptic and extrasynaptic receptors contributing to $GABA_{A,\ slow}$ sIPSCs in CA1 pyramidal cells, we enhanced the spillover component using the selective GABA reuptake inhibitor NO711.

Recordings from CA1 pyramidal cells from wildtype and α123 knock-in mice (Fig. 5A, D) were obtained under baseline condition, then in presence of 2 μM NO711 (Table 4), and finally after application of diazepam (1 μM). To prevent tonic activation of $GABA_B$ receptors (Le Feuvre et al. 1997; Scanziani 2000) 1 μM of the $GABA_B$ antagonist CGP55845 was present throughout the experiment.

Application of 2 μM NO711, greatly enhanced the amplitude and decay time constant of $GABA_{A,\ slow}$ sIPSCs in both genotypes (Fig. 5B, E). In α123 knock-in mice the average amplitude increased from -96.2 ± 8.6 pA to -175.0 ± 32.2 pA and the decay time constant changed from 27.85 ± 18.2 ms to 47.44 ± 2.26 ms ($P < 0.05$, $n = 8$). In wildtype mice, the average amplitude increased from -73.1 ± 6.2 pA to -121.0 ± 19.2 pA and the decay time constant changed from 21.43 ± 1.99 ms to 38.69 ± 2.50ms ($P < 0.05$, $n = 10$). There was also a significant increase in rise time in both genotypes. In contrast, NO711 had no effect on the amplitude and kinetics of $GABA_{A,\ fast}$ sIPSCs (Fig. 5C, F). Subsequent application of diazepam (1 μM) further enhanced amplitude, rise and decay time constant of $GABA_{A,\ slow}$ sIPSCs in both genotypes (Fig. 5B, E). As expected, diazepam also enhanced the amplitude and decay time constant of $GABA_{A,\ fast}$ sIPSCs in wildtype but not in α123 knock-in mice (Fig. 5G, H). In the presence of NO711, no difference between the relative changes in $GABA_{A,\ slow}$ sIPSCs amplitudes in wildtype (1.67 ± 0.20, $n = 10$) and α123 knock-in mice (1.49 ± 0.16, $n = 8$) could be observed after diazepam application.

TABLE 4. ***Baseline properties $GABA_A$ sIPSCs in presence of 1μM CGP 55845***

Genotype and sIPSC type	n	Amplitude, pA	Rise time, ms	Decay time constant, ms
WT, slow	10	-73.1 ± 6.2	15.9 ± 0.9	21.4 ± 2.0
α123, slow	8	-96.2 ± 8.6	16.6 ± 0.6	27.8 ± 1.8
WT, fast	10	-42.1 ± 2.4	0.55 ± 0.02	18.6 ± 1.7
αι23, fast	8	-38.8 ± 2.9	0.61 ± 0.03	14.7 ± 2.6

Values given as means ± SE. For abbreviations, see Table 1.

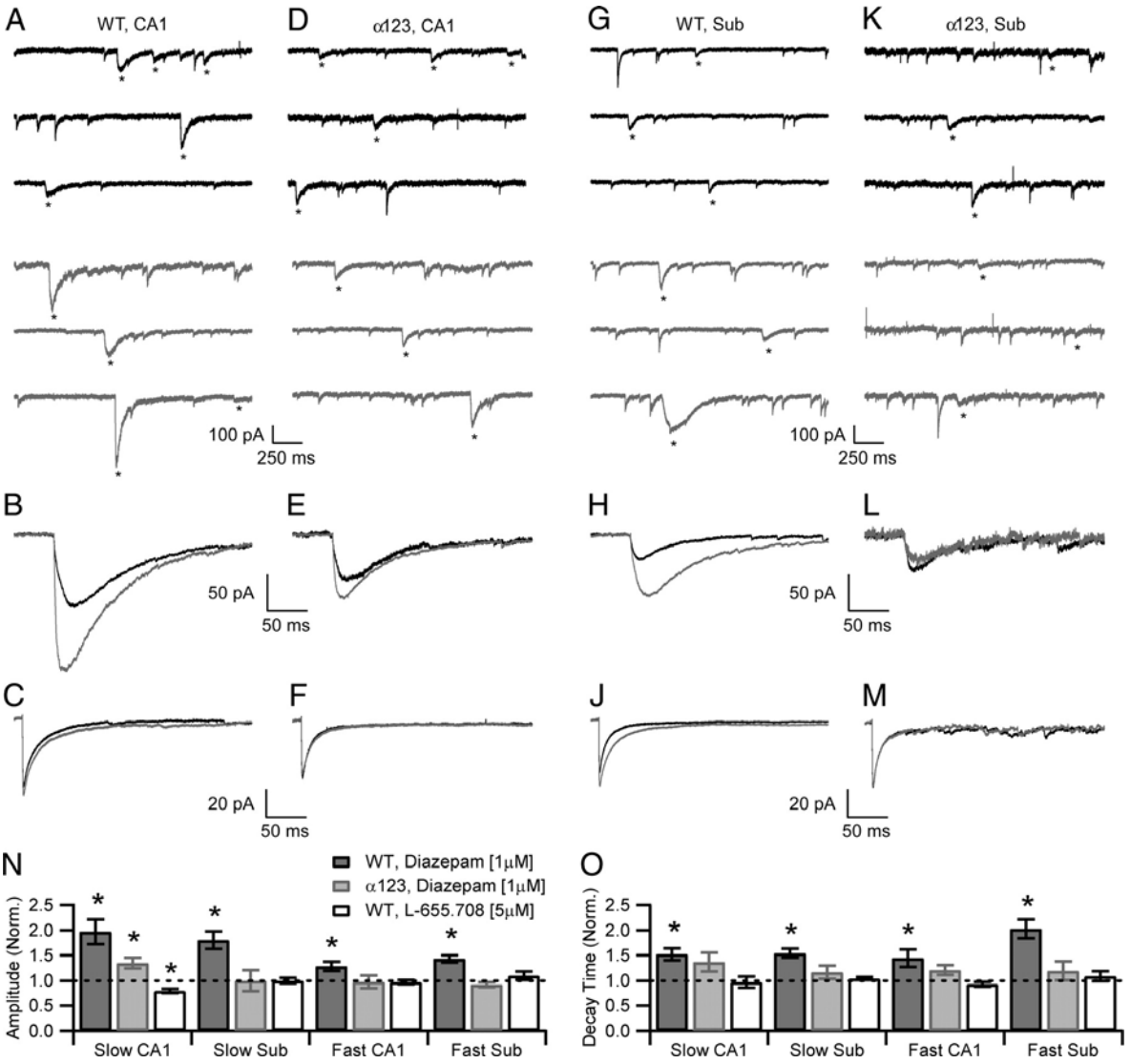


Figure 4. In CA1, but not subicular, pyramidal cells α5-$GABA_A$ receptors ($GABA_A$ receptors) mediate slow spontaneous IPSCs (sIPSCs). Samples of continuous whole-cell voltage-clamp recordings from CA1 (*A* and *D*) and subicular [sub] (*G* and *K*) pyramidal cells in slices obtained from WT (*A* and *G*) and α123 (*D* and *K*) knock-in mice. Baseline conditions in black; gray traces after bath application of 1 μM diazepam. $GABA_{A,slow}$ sIPSCs are marked with asterisks. Corresponding isolated and averaged $GABA_{A,slow}$ sIPSCs (*B*, *E*, *H*, and *L*) and $GABA_{A,fast}$ sIPSCs (*C*, *F*, *J*, and *M*) are shown below with baseline conditions drawn in black and sIPSCs after diazepam application in gray. $GABA_{A,slow}$ sIPSCs can be unambiguously detected in subicular pyramidal cells. In CA1 pyramidal cells, $GABA_{A,slow}$ sIPSC amplitude was increased in WT and α123 knock-in mice after diazepam. In contrast, diazepam had no significant effect on the amplitude of $GABA_{A,slow}$ sIPSCs in the subiculum in α123 knock-in mice, whereas it caused a significant increase in WT mice. *N* and *O*: average normalized effect on amplitudes and decay time constants of sIPSCs in 3 conditions: Diazepam in WT (black bars, $n_{CA1} = 7$, $n_{sub} = 6$), diazepam in α123 knock-in mice (gray bars, $n_{CA1} = 9$, $n_{sub} = 7$), and the α5-$GABA_A$ receptors selective inverse agonist L-655,708 (5 μM) in WT mice (open bars, $n_{CA1} = 7$, $n_{sub} = 5$). *Significant changes from baseline (diazepam, $P < 0.03$; L-655,708, $P < 0.05$, paired Student's *t*-test).

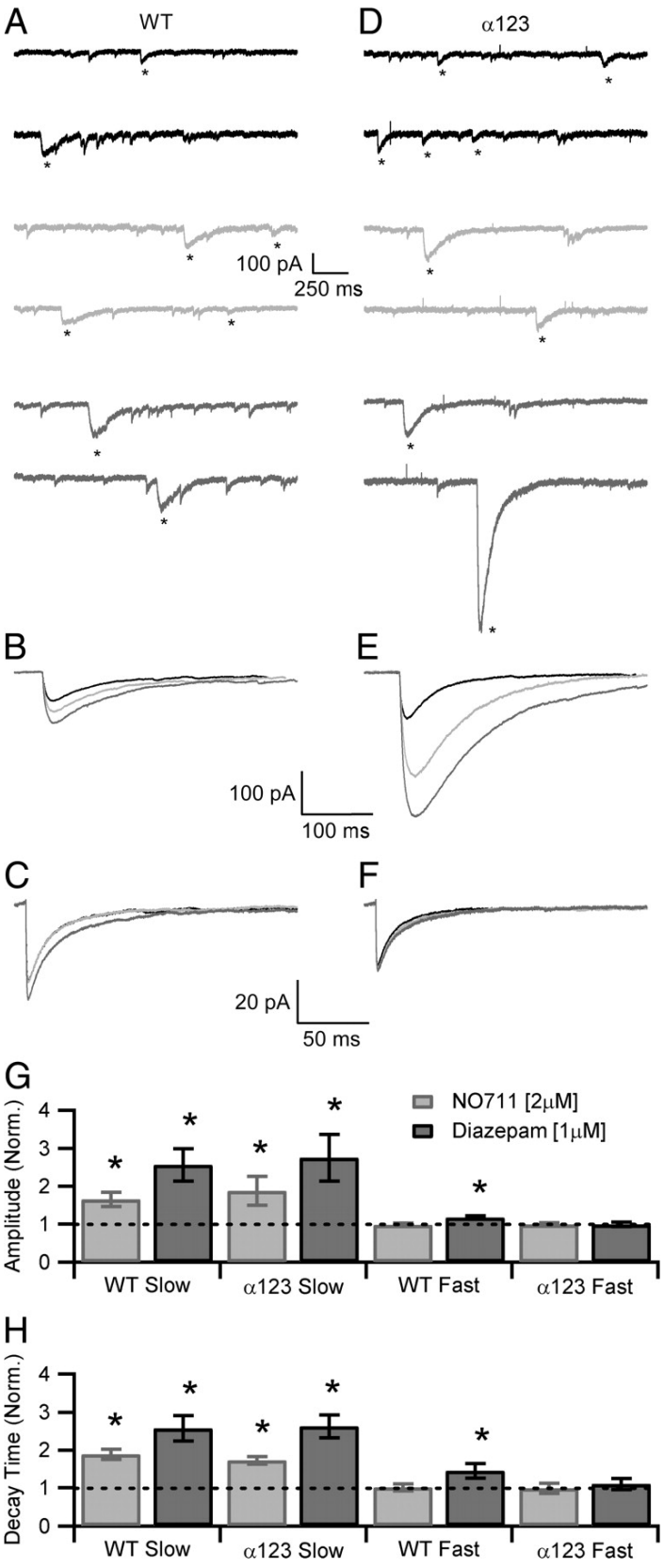


Figure 5. GABA spillover enhances $GABA_{A,slow}$ currents and increases the contribution α5-$GABA_A$ receptors in the CA1 area. *A–F*: sample traces of recordings in the CA1 area in slices from α123 knock-in (*A*) and WT (*D*) mouse in the presence of 1 μM CGP 55845. After baseline recording (black), 2 μM NO711 was added, and the recording was continued (light gray); finally 1 μM diazepam was applied (dark gray). $GABA_{A,slow}$ sIPSCs are marked with an asterisk. Averaged sIPSCs from the α123 knock-in (*B*) and WT (*E*) mouse show typical responses of $GABA_{A,slow}$ sIPSCs to NO711 and the subsequent application of diazepam. Corresponding isolated and averaged $GABA_{A,fast}$ sIPSCs are depicted in *C* and *F* at an enlarged scale to reveal their kinetics. GABA reuptake inhibition increases the amplitude and alters the kinetics only of $GABA_{A,slow}$ sIPSCs. *G* and *H*: comparison of $GABA_{A,slow}$ sIPSCs mean amplitudes (*G*) and mean decay time constants (*H*) normalized to base-line conditions after addition of 2 μM NO711 (light gray) and 1 μM diazepam (dark gray). *Significant difference to preceding condition. Note that diazepam in the presence of NO711 now significantly affects decay time constant of $GABA_{A,slow}$ sIPSCs in α123 knock-in mice ($n = 8$ cells from each genotype, paired Student's *t*-test; $P < 0.05$).

Diazepam activated tonic inhibition in CA1 pyramidal cells is mediated by α5-$GABA_A$ receptors

$GABA_{A,\ slow}$ sIPSCs share elements of tonic GABAergic transmission such as involving perisynaptic $GABA_A$ receptors. Therefore, we assessed which diazepam-sensitive $GABA_A$ receptor subtypes contribute to tonic inhibition in CA1 pyramidal cells. Whole-cell voltage-clamp recordings were made in slices from wildtype and α123 knock-in mice using a high chloride-containing intracellular solution. Using a perfusion rate of 1 ml/min, tonic inhibition was measured as the change in holding current after the application 100 μM picrotoxin in the presence of the $GABA_B$ antagonist CGP 55845 (1 μM). No GABA reuptake inhibitor was used. Under these conditions, application of 100 μM picrotoxin produced an outward shift in the baseline current of 33 ± 4 pA (n = 7) at room temperature and 35 ± 9 pA (n = 5) at physiological temperature. The tonic conductance was mediated by $GABA_A$ receptors because it was also blocked by bicuculline (10 μM) (data not shown). Since it has been reported that low doses of picrotoxin selectively (e.g. more rapidly) block tonic inhibition (Semyanov et al. 2003), we performed the same experiments using 2 μM picrotoxin and measured the outward shift of the holding current during two distinct time windows: at 120 - 150 seconds (t1) and 240 - 300 seconds (t2) after picrotoxin application. The holding current at t1 changed by 22 ± 5 pA (n = 6) (Fig. 6A, B middle trace, and C) and by 33 ± 4 pA at t2 (n = 6) (Fig. 6A, B right trace, and C). RMS noise decreased from 5.4 ± 0.27 pA to 4.4 ± 0.27 pA ($P < 0.05$) at t1 and to 3.9 ± 0.18 pA, (n.s.) at t2. The amplitude of fast events remained constant at t1, whereas a tendency for smaller $GABA_{slow}$ amplitudes was observed at that time point. At time t2 both fast and slow events were significantly reduced in amplitude (–44.8 ± 1.1 to -28.6 ± 0.65 pA, n=6, $P < 0.01$ in $_{fast}$ sIPSCs and –61.1 ± 7.5 to –34.4 ± 1.9 pA, n=6, $P < 0.01$ in $_{slow}$ sIPSCs) (Fig. 6 D). At t1, the frequency and decay time constant of $GABA_{A,\ fast}$ sIPSCs were unaltered compared to baseline (3.8 ± 0.26 Hz to 4.0 ± 0.17 Hz, n=6; and 21.7 ± 0.7 to 23.1 ± 0.9 ms, respectively) (Fig. 6E and 6F, left trace), whereas the frequency and decay time of $GABA_{A,\ slow}$ sIPSCs were significantly reduced (from 0.09 ± 0.01 Hz to 0.06 ± 0.01 Hz n = 6, $P < 0.05$ and from 27.6 ± 2.3 to 20.2 ± 2.8 ms, $P < 0.05$, respectively) (Fig. 6E and 6F, right trace). The selective change in decay time and frequency of $GABA_{A,\ slow}$ sIPSCs which correlated with the change in holding current, provides further indication

that slow events and tonic inhibition share a common pool of extrasynaptic and thus most likely α5-$GABA_A$ receptors (Caraiscos et al. 2004a).

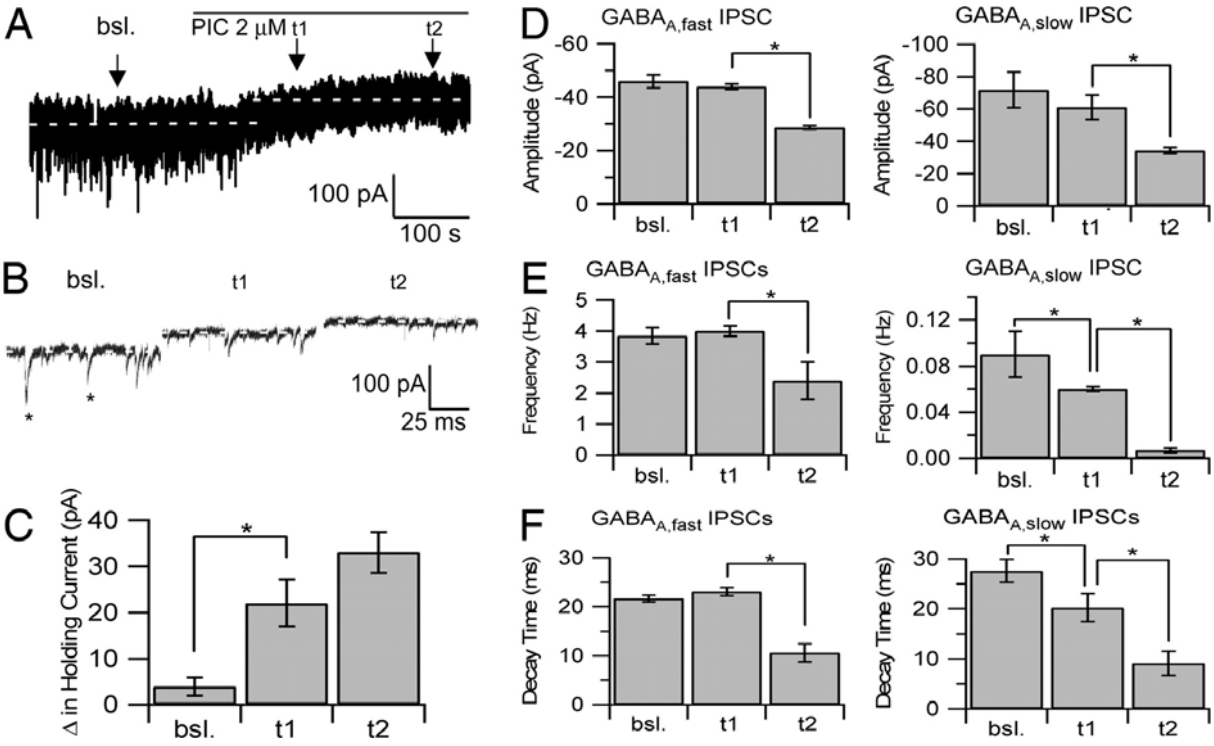


Figure 6. Effect of picrotoxin (PIC) on holding current and $GABA_{A,fast}$ and $GABA_{A,slow}$ sIPSCs. *A*: representative trace from a continuous voltage-clamp recording at room temperature from a CA1 pyramidal cell of a WT mouse. PIC (2 µM) application to bath solution resulted in a decrease in the inward holding current and a reduction in the frequency and decay time of sIPSCs. The arrows marked t1 and t2 represent the 2 time windows during which holding current and spontaneous events were analyzed. *B*: magnified 1-s traces of baseline (*left*), t1 (*middle*), and t2 (*right*). $GABA_{A,slow}$ sIPSCs are marked with asterisks. *C*: average change in holding current measured at t1 and at t2; holding current was significantly reduced at t1 (*$P < 0.05$, $n = 6$, paired Student's *t*-test). *D*: average peak amplitude in fast (*left*) and slow sIPSCs (*right*); amplitude of fast and slow events was significantly reduced at t2 (*$P < 0.006$, $n = 6$, paired Student's *t*-test). *E*: averaged change in frequency of fast (*left*) and slow (*right*) sIPSCs. At t1, as the holding current was significantly reduced, fast sIPSC frequency was unaltered (*left*), whereas frequency of slow sIPSCs was significantly reduced (*right*; *$P < 0.05$, $n = 6$, paired Student's *t*-test). ANOVA shows significant differences between baseline, t1, and t2 ($n = 6$, $P \leq 0.01$). *F*: averaged change in decay time of fast (*left*) and slow (i) sIPSCs. Changes follow the pattern seen in *D* and *E*. At t1, fast sIPSC decay time was unaltered (*left*), whereas decay time of slow sIPSCs was significantly reduced (*right*; *$P < 0.05$, $n = 6$, paired Student's *t*-test). ANOVA reveals significant differences between baseline, t1, and t2 ($n = 6$, $P \leq 0.01$).

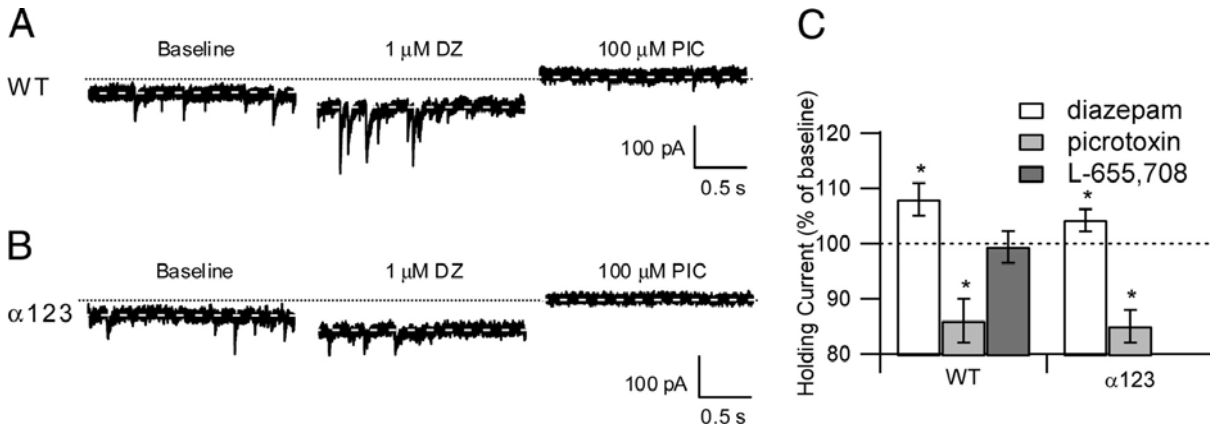


Figure 7. Effect of diazepam and L-655,708 on tonic current from CA1 pyramidal cells. *A*: representative trace segments recorded in a CA1 pyramidal cell from WT mouse. Note the inward drift in the holding current after the application of 1 μM diazepam as well as the increase in amplitude and frequency from spontaneous events (*middle*). After 5 min of PIC (100 μM) application to bath solution, holding current had an outward drift equal to that observed with 2 μM PIC. *B*: representative traces from a recorded CA1 pyramidal cell in α123 knock-in mouse. After application of 1 μM diazepam to the bath solution, holding current exhibited an inward drift, but frequency and amplitude of fast spontaneous events remained unchanged (*middle*). Outward drift in holding current after application of PIC (100 μM) was similar as in WT mice. *C*: average shift in holding current in percentage of baseline after drug applications: In WT mice, holding current increased after diazepam application by 12 ± 7 pA (n = 10), whereas in α123 knock-in mice, it increased by 7 ± 2 pA (n = 6). Increase in holding current after diazepam application showed no significant difference between WT and α123 knock-in mice (unpaired Student's t-test, not significant). When using 5 μM L-655,708 instead of diazepam, no effect on holding current from CA1 pyramidal cells was observed (n = 4). Subsequent application of PIC (100 μM) in these cells caused a change in holding current comparable with diazepam experiments. L-655,708 was not used on α123 knock-in mice. *Significant changes from baseline ($P < 0.05$, 2-tailed paired Student's t-test).

Supporting this hypothesis, application of diazepam caused an inward shift in baseline holding current recorded in wildtype mice (10 ± 4 pA, n = 7, Fig. 7A). In α123 knock-in mice, a similar inward shift in baseline holding current was also observed after the application of 1 μM diazepam (8 ± 2 pA, n = 5) (Fig. 7B, C), demonstrating that α5-$GABA_A$ receptors mediate the diazepam-sensitive component of tonic inhibition in CA1 pyramidal cells. The application of 5 μM L-655,708 instead of diazepam in on pyramidal cells of the CA1 area from wildtype mice did not significantly alter the holding current (n = 4) (Fig. 7C), indicating that diazepam application increased the affinity of previously silent α5-$GABA_A$ receptors to the point that they were activated by ambient GABA. As no reliable electrophysiological data on the affinity and activity of L-655,708 on $GABA_A$

receptors carrying point mutated α-subunits is available we only used this substance in wildtype mice.

Discussion

The present results demonstrate clear task specificity for $GABA_A$ receptors subtypes distinguished by their α subunit composition in the hippocampal formation. In CA1 pyramidal cells, fast synaptic inhibition is mediated selectively by α1- and α2- $GABA_A$ receptors, with a spatial segregation of the two different receptor subtypes in distal and proximal compartments, respectively. Slow phasic inhibition involves both synaptic and extrasynaptic receptors, the latter being principally α5-$GABA_A$ receptors. Finally, diazepam-sensitive tonic inhibition, which can be observed in the absence of GABA reuptake inhibitors, is mediated by α5-$GABA_A$ receptors. In subiculum, where the α5 subunit is expressed at very low levels, $GABA_{A,\ slow}$ sIPSCs can be readily detected, indicating that these synaptic events do not depend on α5-$GABA_A$ receptors. Nevertheless, a mixture of synaptic and extrasynaptic receptors also mediates them. Altogether, these results indicate that the distinct modes of synaptic inhibition in hippocampal neurons involve different $GABA_A$ receptor subtypes, organized in a region-specific manner and precisely targeted to distinct synaptic, perisynaptic, and extrasynaptic sites.

Technical considerations

The conclusions of this study are derived from a pharmacological distinction of $GABA_A$ receptor subtypes in knock-in mutant mice. There are two major prerequisites to validate these conclusions. First, the point-mutation should not alter the organization and functional properties of $GABA_A$ receptors in mutant mice. Several lines of evidence indicate that this prerequisite is fulfilled: Animals containing one or more point-mutations develop normally, have no overt phenotype and the cellular and subcellular location of $GABA_A$ receptors is unchanged (Benke et al. 2004; Mohler et al. 2004; Rudolph et al. 1999, 2004; Yee et al. 2004). The functional properties of receptors containing mutated subunits are unaltered *in vivo* (Bacci et al. 2003; Fagiolini et al. 2004; Marowsky et al. 2004). The present results reveal no difference across genotypes in the kinetics of sIPSCs or eIPSCs, and in the stimulus strength required to evoke IPSCs of similar amplitude, confirming that functional properties of $GABA_A$ receptors are normal in mutant mice. Second, the effects

of diazepam on peak amplitude and/or decay time constant should be detectable on all sensitive $GABA_A$ receptor subtypes and should not be masked by agonist saturation. At least for the α1, α2, and α5-$GABA_A$ receptors these conditions were fulfilled, confirming that mouse CA1 pyramidal cells have an incomplete postsynaptic $GABA_A$ receptor occupancy at room temperature (Hajos et al. 2000; Perrais and Ropert 2000, 1999). Failure to detect a contribution of α3-$GABA_A$ receptors to eIPSCs likely reflects the low abundance of these receptors in CA1 pyramidal cells (Laurie et al. 1992) (Fig. 1). Therefore, with the knock-in strategy, the function of $GABA_A$ receptors containing specific αsubunits can be analyzed with great selectivity (Rudolph and Mohler 2004) and without inducing compensatory changes, as occurs in gene deletion experiments. To date, no drugs are available to discriminate between α1-, α2-, and α3-$GABA_A$ receptors *in vivo* with a comparable degree of selectivity. One obvious limitation of the approach lies in the fact that it is limited to diazepam-sensitive $GABA_A$ receptors.

Somatic and dendritic inputs are mediated differentially by α2- and α1- $GABA_A$ receptors

The results from the analysis of eIPSCs are largely in agreement with the known subcellular distribution of $GABA_A$ receptor subtypes in CA1 pyramidal cells. α2-$GABA_A$ receptors located predominantly on the axon-initial segment and on the soma, facing terminals from chandelier cells and from basket cells expressing cholecystokinin, respectively (Nusser et al. 1996; Nyiri et al. 2001). The α1-$GABA_A$ receptors on the soma are contacted by parvalbumin-positive basket cell terminals, and they predominate in the dendritic layers of CA1, as seen by immunohistochemistry (Fig. 1) (Brunig et al. 2002). This segregation of different $GABA_A$ receptors to different subcellular compartments is of functional relevance, as demonstrated *in vivo* with the contribution of distinct interneurons signaling through these receptors during specific behavioral states (Klausberger 2002, 2003). Additionally, Pouille and Scanziani (2001, 2004) have recently demonstrated that feedforward and feedback inhibition are mediated predominantly by somatic and dendritic receptors respectively. The differential modulation by diazepam of IPSCs evoked by proximal and distal stimulation of inhibitory inputs in α1 and α2 knock-in mice confirms this functional segregation of α2- and α1-$GABA_A$ receptors, thereby validating the use of this knock-in model to pharmacologically isolate specific $GABA_A$ receptor subtypes. The agreement between the present results and the known segregation of α1- and α2-$GABA_A$ receptors in CA1 neurons makes it unlikely that the difference in the effects of diazepam

on amplitude of eIPSCs in α1 and α2 knock-in mice are due to differential occupancy of receptors located on the soma and dendrites. To explain the present results, the degree of occupancy of these receptors should be opposite on the soma and dendrites (α1 high on soma and low on dendrites, and vice-versa for α2), which appears rather unlikely. Finally, it is well established that α2-$GABA_A$ receptors deactivate more slowly than α1-$GABA_A$ receptors, as shown in recombinant systems (McClellan et al. 1999) and *in vivo* (Bosman et al. 2005; Goldstein et al. 2002; Vicini et al. 2001). This distinction was not apparent with eIPSCs, reflecting the detection of compound, non-synchronous events and the variable effects of dendritic filtering upon proximal and distal stimulation.

$GABA_{A, slow}$ IPSCs involve activation of synaptic and perisynaptic receptors

$GABA_{A, slow}$ IPSCs in CA1 pyramidal cells have been observed in evoked, spontaneous, and miniature IPSCs (Banks et al. 1998; Pearce 1993) $GABA_{A, slow}$ eIPSCs exhibit the same properties as $GABA_{A, slow}$ sIPSCs and probably emanate from the same synapse (Banks et al. 1998). Our data extend the previous findings by demonstrating the presence of $GABA_{A, slow}$ sIPSCs also in the subiculum. It was not possible to determine whether $GABA_{A, slow}$ IPSCs in CA1 and subicular pyramidal cells are produced by similar mechanisms. The question whether $GABA_{A, slow}$ IPSCs are produced by one specific cell type common to both brain areas therefore remains unresolved.

Slow events have been proposed to involve phasic activation of perisynaptic or even extrasynaptic $GABA_A$ receptors upon GABA spillover from the synaptic cleft (Banks et al. 1998). The sensitivity of $GABA_{A, slow}$ to diazepam in α123 knock-in mice and their depression by L-655,708 in wildtype mice demonstrate that α5-$GABA_A$ receptors contribute to $GABA_{A, slow}$ sIPSCs. More importantly, this contribution is amplified and likely dominates the current in conditions of enhanced spillover, since the diazepam induced increase in $GABA_{A, slow}$ currents became indistinguishable between wildtype and α123 knock-in mice in presence of a GABA uptake inhibitor. An enhanced diffusion volume covered by the neurotransmitter and the prolonged gating kinetics of the receptors by the elevated concentration of GABA can explain the change in rise and decay time constant of $GABA_{A, slow}$ in the presence of a GABA uptake inhibitor. Which particular mechanism prevails would most likely be determined by the amount of spilled out GABA.

Our results clearly show that the spillover component of slow phasic currents is mediated by α5-$GABA_A$ receptors and nicely agree with the predominantly extrasynaptic distribution of α5-$GABA_A$ receptors (Brunig et al. 2002; Caraiscos et al. 2004a,b; Crestani et al. 2002). Interestingly, however, $GABA_{A, slow}$ sIPSCs also occurred in subiculum where the α5 subunit is virtually absent. The potentiation by diazepam in wildtype mice provides direct evidence that $GABA_{A, slow}$ sIPSCs there are also mediated by other diazepam-sensitive $GABA_A$ receptor subtypes. These IPSCs are otherwise indistinguishable from those recorded in CA1, suggesting that the function of α5-$GABA_A$ receptors is taken over by other $GABA_A$ receptors with similar peri- and extrasynaptic localization in subicular neurons.

The stronger effect of diazepam on $GABA_{A, slow}$ sIPSCs in wildtype compared to α123 knock-in mice in CA1 pyramidal cells and the partial effect of L-655,708 (Fig 4) implicate the involvement of additional $GABA_A$ receptor subtypes in slow currents, with α1-$GABA_A$ receptors as likely co-participants, as this subunit has been demonstrated sometimes to co-localize with the α5 subunit in the same clusters (Hutcheon et al. 2004). The lack of diazepam effect on $GABA_{A, fast}$ sIPSCs in α123 knock-in mice (Fig. 4) indicates that α5-$GABA_A$ receptors do not participate in fast phasic inhibition. We did not detect any spillover component in fast GABAergic sIPSCs in the CA1 region and even the combination of NO711 and diazepam on cells from α123 mice revealed no contribution of α5-$GABA_A$ receptors to $GABA_{A, fast}$. Therefore, these $GABA_A$ receptors are targeted selectively to sites mediating $GABA_{A, slow}$ sIPSCs in CA1 pyramidal cells, where they are probably located both in the synaptic cleft and perisynaptically. Using paired intracellular recordings; Thomson et al. (2000) provided indirect pharmacological evidence for the existence of α5-$GABA_A$ receptors mediating fast phasic inhibition from bistratified cells, which innervate distal pyramidal cell dendrites. The failure to detect these receptors in point-mutated mice might reflect the minor contribution of these events to the overall number of sIPSCs received by pyramidal cells. Banks *et al.* (1998) provided evidence for blockade of $GABA_{A, fast}$ sIPSCs by furosemide, suggesting a contribution of α4-$GABA_A$ receptors to these events. We did not assess the contribution of α4-$GABA_A$ receptors, which are diazepam-insensitive, but their low expression level in CA1 area (Pirker et al. 2000) makes it unlikely that they play a major role in mediating $GABA_{A, fast}$ sIPSCs.

Our findings emphasize the proposed role of α5-$GABA_A$ receptors as detectors for extrasynaptic GABA (Caraiscos et al. 2004a; Glykys and Mody, 2006), and reveal their dual assignment to the generation of slow GABAergic events and the mediation of diazepam-sensitive tonic inhibition. In both cases GABA is not confined to synaptic clefts and the extent to which the neurotransmitter release underlying $GABA_{A,\ slow}$ sIPSCs contributes to ambient GABA remains to be examined.

Tonic inhibition

So far we have shown that in the CA1 area, $GABA_{A,\ slow}$ events partially arise from the activation of α5-$GABA_A$ receptors that are predominantly located extrasynaptically. Since $GABA_{A,\ slow}$ sIPSCs are rare, their early block by the activity-dependent antagonist picrotoxin, which parallels the early decrease of tonic GABAergic currents, most likely reflects crosstalk between the receptor populations mediating these two types of GABAergic currents. The pronounced sensitivity of tonic inhibition and $GABA_{A,\ slow}$ for picrotoxin is in line with a higher opening probability of tonically activated, extrasynaptic receptors.

The increase in holding current after application of diazepam in both wildtype and α123 knock-in mice confirms that α5-$GABA_A$ receptors can generate both tonic inhibition and slow events in CA1 pyramidal cells. Several lines of evidence indicate that most α5-$GABA_A$ receptors contain the β3 subunit in pyramidal neurons and β3-$GABA_A$ receptors are present in extrasynaptic regions (Pirker et al. 2000). It would be of major interest to know whether these receptors have the required high affinity for GABA. The fact that in our experiments the inverse agonist L-655,708 in contrast to diazepam, failed to exert any effect on the holding current of CA1 pyramidal cells raises questions about the role of α5-$GABA_A$ receptors detecting ambient GABA concentrations in the absence of diazepam. Diazepam increases the probability of $GABA_A$ receptors to be activated after GABA binding. This can lead to tonic opening of receptors under low ambient GABA concentration. Consequently, an inverse agonist fails to produce any effect on previously not activated receptors. Scimemi et al. (2005) have been the first to address the dependency of α5-$GABA_A$ receptors activation on GABA concentrations. The authors found that L-655,708 decreases the holding current only when ambient GABA concentrations are elevated. Under physiological conditions, synchronous neural network activity might lead to such heightened ambient GABA concentration and subsequent activation of

extracellular α5-$GABA_A$ receptors (Towers et al. 2004). Nevertheless our findings are relevant to explain the physiological effects of diazepam, as this drug experiences widespread pharmaceutical use.

Significance of the work

We have shown that the subunit composition of $GABA_A$ receptors not only affects their subcellular distribution but also dictates their functional role. The clear segregation of $GABA_A$ receptor subtypes demonstrated here shows the complex organization of the inhibitory system at the molecular level and provides a compelling explanation for the specific effects of different $GABA_A$ receptors in distinct behaviorally significant signaling pathways.

Acknowledgments

We thank Corinne Sidler and Franziska Parpan for their excellent technical assistance.

Grants

G.A. Prenosil, E. M. Schneider Gasser and K.E. Vogt are supported by

grant # 631-066012 of the Swiss National Science Foundation.

References

Bacci A, Rudolph U, Huguenard JR, and Prince DA. Major differences in inhibitory synaptic transmission onto two neocortical interneuron subclasses. *J Neurosci* 23: 9664-9674, 2003.

Banks MI, Hardie JB, and Pearce RA. Development of $GABA_A$ receptor-mediated inhibitory postsynaptic currents in hippocampus. *J Neurophysiol* 88: 3097-3107, 2002.

Banks MI, Li TB, and Pearce RA. The synaptic basis of $GABA_A$,slow. *J Neurosci* 18: 1305-1317, 1998.

Banks MI and Pearce RA. Kinetic differences between synaptic and extrasynaptic $GABA_A$ receptors in CA1 pyramidal cells. *J Neurosci* 20: 937-948, 2000.

Banks MI, White JA, and Pearce RA. Interactions between distinct $GABA_A$ circuits in hippocampus. *Neuron* 25: 449-457, 2000.

Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN, and Langer SZ. International Union of Pharmacology. XV. Subtypes of $GABA_A$ receptors: classification on the basis of subunit structure and receptor function. *Pharmacol Rev* 50: 291-313, 1998.

Benke D, Fakitsas P, Roggenmoser C, Michel C, Rudolph U, and Mohler H. Analysis of the presence and abundance of $GABA_A$ receptors containing two different types of α subunits in murine brain using point-mutated α subunits. *J Biol Chem* 279: 43654-43660, 2004.

Benson JA, Low K, Keist R, Mohler H, and Rudolph U. Pharmacology of recombinant $GABA_A$ receptors rendered diazepam-insensitive by point-mutated α-subunits. *FEBS Lett* 431: 400-404, 1998.

Bosman LW, Heinen K, Spijker S, and Brussaard AB. Mice lacking the major adult $GABA_A$ receptor subtype have normal number of synapses, but retain juvenile IPSC kinetics until adulthood. *J Neurophysiol* 94: 338-346, 2005.

Bosman LW, Rosahl TW, and Brussaard AB. Neonatal development of the rat visual cortex: synaptic function of $GABA_A$ receptor α subunits. *J Physiol* 545: 169-181, 2002.

Brickley SG, Cull-Candy SG, and Farrant M. Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of $GABA_A$ receptors. *J Physiol* 497: 753-759, 1996.

Brunig I, Scotti E, Sidler C, and Fritschy JM. Intact sorting, targeting, and clustering of γ-aminobutyric acid A receptor subtypes in hippocampal neurons *in vitro*. *J Comp Neurol* 443: 43-55, 2002.

Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF, and Orser BA. Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing γ-aminobutyric acid type A receptors. *Proc Natl Acad Sci U S A* 101: 3662-3667, 2004a.

Caraiscos VB, Newell JG, You-Ten KE, Elliott EM, Rosahl TW, Wafford KA, MacDonald JF, and Orser BA. Selective enhancement of tonic GABAergic inhibition in murine hippocampal neurons by low concentrations of the volatile anesthetic isoflurane. *J Neurosci* 24: 8454-8458, 2004b.

Cope DW, Hughes SW, Crunelli V. $GABA_A$ receptor-mediated tonic inhibition in thalamic neurons. *J Neurosci* 25:11553-11563, 2005.

Crestani F, Keist R, Fritschy JM, Benke D, Vogt K, Prut L, Bluthmann H, Mohler H, and Rudolph U. Trace fear conditioning involves hippocampal α5-$GABA_A$ receptors. *Proc Natl Acad Sci U S A* 99: 8980-8985, 2002.

Drasbek KR, Jensen K. THIP, a hypnotic and antinociceptive drug, enhances an extrasynaptic $GABA_A$ receptor-mediated conductance in mouse neocortex. *Cereb Cortex* Oct 12, 2005; 10.1093/cercor/bhj055

Fagiolini M, Fritschy JM, Low K, Mohler H, Rudolph U, and Hensch TK. Specific $GABA_A$ circuits for visual cortical plasticity. *Science* 303: 1681-1683, 2004.

Farrant M and Nusser Z. Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. *Nat Rev Neurosci* 6: 215-229, 2005.

Freund TF and Buzsaki G. Interneurons of the hippocampus. *Hippocampus* 6: 347-470, 1996.

Fritschy JM and Mohler H. $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J Comp Neurol* 359: 154-194, 1995.

Fritschy JM, Weinmann O, Wenzel A, and Benke D. Synapse-specific localization of NMDA and $GABA_A$ receptor subunits revealed by antigen-retrieval immunohistochemistry. *J Comp Neurol* 390: 194-210, 1998.

Glykys J, Mody I. Hippocampal network hyperactivity following selective reduction of tonic inhibition in $GABA_A$ receptor α5 subunit deficient mice, *J Neurophysiol* Feb 1, 2006; 10.1152/jn.01122.2005.

Goldstein PA, Elsen FP, Ying SW, Ferguson C, Homanics GE, and Harrison NL. Prolongation of hippocampal miniature inhibitory postsynaptic currents in mice lacking the $GABA_A$ receptor α1 subunit. *J Neurophysiol* 88: 3208-3217, 2002.

Hajos N, Nusser Z, Rancz EA, Freund TF, and Mody I. Cell-type- and synapse-specific variability in synaptic $GABA_A$ receptor occupancy. *Eur J Neurosci* 12: 810-818, 2000.

Hutcheon B, Fritschy JM, and Poulter MO. Organization of $GABA_A$ receptor α-subunit clustering in the developing rat neocortex and hippocampus. *Eur J Neurosci* 19: 2475-2487, 2004.

Hutcheon B, Morley P, and Poulter MO. Developmental change in $GABA_A$ receptor desensitization kinetics and its role in synapse function in rat cortical neurons. *J Physiol* 522: 3-17, 2000.

Klausberger T. Brain-state- and cell type-specific firing of hippocampal interneurons *in vivo*. *Nature* 421: 844-848, 2003.

Klausberger T, Roberts JD, and Somogyi P. Cell type- and input-specific differences in the number and subtypes of synaptic $GABA_A$ receptors in the hippocampus. *J Neurosci* 22: 2513-2521, 2002.

Knoflach F, Rhyner T, Villa M, Kellenberger S, Drescher U, Malherbe P, Sigel E, and Mohler H. The γ3-subunit of the $GABA_A$-receptor confers sensitivity to benzodiazepine receptor ligands. *FEBS Lett* 293: 191-194, 1991.

Laurie DJ, Wisden W, and Seeburg PH. The distribution of thirteen $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci* 12: 4151-4172, 1992.

Le Feuvre Y, Fricker D, and Leresche N. $GABA_A$ receptor-mediated IPSCs in rat thalamic sensory nuclei: patterns of discharge and tonic modulation by $GABA_B$ autoreceptors. *J Physiol* 502: 91-104, 1997.

Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, Fritschy JM, Rulicke T, Bluethmann H, Mohler H, and Rudolph U. Molecular and neuronal substrate for the selective attenuation of anxiety. *Science* 290: 131-134, 2000.

Marowsky A, Fritschy JM, and Vogt KE. Functional mapping of $GABA_A$ receptor subtypes in the amygdala. *Eur J Neurosci* 20: 1281-1289, 2004.

McClellan AM, and Twyman RE. Receptor system response kinetics reveal functional subtypes of native murine and recombinant human $GABA_A$ receptors. *J Physiol* 515: 711-727, 1999.

McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, Farrar S, Myers J, Cook G, Ferris P, Garrett L, Bristow L, Marshall G, Macaulay A, Brown N, Howell O, Moore KW, Carling RW, Street LJ, Castro JL, Ragan CI, Dawson GR, and Whiting PJ. Sedative but not anxiolytic properties of benzodiazepines are mediated by the $GABA_A$ receptor α1 subtype. *Nat Neurosci* 3: 587-592, 2000.

McKernan RM and Whiting PJ. Which $GABA_A$-receptor subtypes really occur in the brain? *Trends Neurosci* 19: 139-143, 1996.

Mtchedlishvili Z, Kapur J. High-affinity, slowly desensitizing $GABA_A$ receptors mediate tonic inhibition in hippocampal dentate granule cells. *Mol Pharmacol* 69:564-575, 2006.

Mody I and Pearce RA. Diversity of inhibitory neurotransmission through $GABA_A$ receptors. *Trends Neurosci* 27: 569-575, 2004.

Mohler H, Fritschy JM, Crestani F, Hensch T, and Rudolph U. Specific $GABA_A$ circuits in brain development and therapy. *Biochem Pharmacol* 68: 1685-1690, 2004.

Mohler H, Fritschy JM, and Rudolph U. A new benzodiazepine pharmacology. *J Pharmacol Exp Ther* 300: 2-8, 2002.

Nusser Z and Mody I. Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. *J Neurophysiol* 87: 2624-2628, 2002.

Nusser Z, Sieghart W, Benke D, Fritschy JM, and Somogyi P. Differential synaptic localization of two major $GABA_A$ receptor α subunits on hippocampal pyramidal cells. *Proc Natl Acad Sci U S A* 93: 11939-11944, 1996.

Nyiri G, Freund TF, and Somogyi P. Input-dependent synaptic targeting of α2-subunit-containing $GABA_A$ receptors in synapses of hippocampal pyramidal cells of the rat. *Eur J Neurosci* 13: 428-442, 2001.

Pearce RA. Physiological evidence for two distinct $GABA_A$ responses in rat hippocampus. *Neuron* 10: 189-200, 1993.

Perrais D and Ropert N. Altering the concentration of GABA in the synaptic cleft potentiates miniature IPSCs in rat occipital cortex. *Eur J Neurosci* 12: 400-404, 2000.

Perrais D and Ropert N. Effect of zolpidem on miniature IPSCs and occupancy of postsynaptic $GABA_A$ receptors in central synapses. *J Neurosci* 19: 578-588, 1999.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, and Sperk G. $GABA_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience* 101: 815-850, 2000.

Porcello DM, Huntsman MM, Mihalek RM, Homanics GE, and Huguenard JR. Intact synaptic GABAergic inhibition and altered neurosteroid modulation of thalamic relay neurons in mice lacking δ subunit. *J Neurophysiol* 89: 1378-1386, 2003.

Pouille F and Scanziani M. Enforcement of temporal fidelity in pyramidal cells by somatic feed-forward inhibition. *Science* 293: 1159-1163, 2001.

Pouille F and Scanziani M. Routing of spike series by dynamic circuits in the hippocampus. *Nature* 429: 717-723, 2004.

Price CJ, Cauli B, Kovacs ER, Kulik A, Lambolez B, Shigemoto R, and Capogna M. Neurogliaform neurons form a novel inhibitory network in the hippocampal CA1 area. *J Neurosci* 25: 6775-6786, 2005.

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, and Seeburg PH. Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature* 338: 582-585, 1989.

Quirk K, Blurton P, Fletcher S, Leeson P, Tang F, Mellilo D, Ragan CI, McKernan RM. [^{3}H]L-655,708, a novel ligand selective for the benzodiazepine site of $GABA_A$ receptors which contain the α5 subunit. *Neuropharmacology* 35: 1331-1335, 1996

Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, and Mohler H. Benzodiazepine actions mediated by specific $GABA_A$ receptor subtypes. *Nature* 401: 796-800, 1999.

Rudolph U and Mohler H. Analysis of $GABA_A$ receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. *Annu Rev Pharmacol Toxicol* 44: 475-498, 2004.

Scanziani M. GABA spillover activates postsynaptic $GABA_B$ receptors to control rhythmic hippocampal activity. *Neuron* 25: 673-681, 2000.

Scholze P, Ebert V, and Sieghart W. Affinity of various ligands for $GABA_A$ receptors containing α4β3γ2, α4γ2, or α1β3γ2 subunits. *Eur J Pharmacol* 304: 155-162, 1996.

Scimemi A, Semyanov A, Sperk G, Kullmann DM, and Walker MC. Multiple and plastic receptors mediate tonic $GABA_A$ receptor currents in the hippocampus. *J Neurosci* 25: 10016-10024, 2005.

Semyanov A, Walker MC, and Kullmann DM. GABA uptake regulates cortical excitability via cell type-specific tonic inhibition. *Nat Neurosci* 6: 484-490, 2003.

Semyanov A, Walker MC, Kullmann DM, and Silver RA. Tonically active $GABA_A$ receptors: modulating gain and maintaining the tone. *Trends Neurosci* 27: 262-269, 2004.

Sieghart W and Sperk G. Subunit composition, distribution and function of $GABA_A$ receptor subtypes. *Curr Top Med Chem* 2: 795-816, 2002.

Smith AJ, Alder L, Silk J, Adkins C, Fletcher AE, Scales T, Kerby J, Marshall G, Wafford KA, McKernan RM, and Atack JR. Effect of α subunit on allosteric modulation of ion channel function in stably expressed human recombinant $GABA_A$ receptors determined using ^{36}Cl ion flux. *Mol Pharmacol* 59: 1108-1118, 2001.

Stell BM, Brickley SG, Tang CY, Farrant M, and Mody I. Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subunit-containing $GABA_A$ receptors. *Proc Natl Acad Sci U S A* 100: 14439-14444, 2003.

Sun C, Sieghart W, and Kapur J. Distribution of α1, α4, γ2, and δ subunits of $GABA_A$ receptors in hippocampal granule cells. *Brain Res* 1029: 207-216, 2004.

Thomson AM, Bannister AP, Hughes DI, and Pawelzik H. Differential sensitivity to Zolpidem of IPSPs activated by morphologically identified CA1 interneurons in slices of rat hippocampus. *Eur J Neurosci* 12: 425-436, 2000.

Towers SK, Gloveli T, Traub RD, Driver JE, Engel D, Fradley R, Rosahl TW, Maubach K, Buhl EH, and Whittington MA. α5 subunit-containing $GABA_A$ receptors affect the dynamic range of mouse hippocampal kainate-induced γ frequency oscillations *in vitro*. *J Physiol* 559: 721-728, 2004.

Vicini S, Ferguson C, Prybylowski K, Kralic J, Morrow AL, and Homanics GE. $GABA_A$ receptor α1 subunit deletion prevents developmental changes of inhibitory synaptic currents in cerebellar neurons. *J Neurosci* 21: 3009-3016, 2001.

White JA, Banks MI, Pearce RA, and Kopell NJ. Networks of interneurons with fast and slow γ-aminobutyric acid type A ($GABA_A$) kinetics provide substrate for mixed γ-τ rhythm. *Proc Natl Acad Sci U S A* 97: 8128-8133, 2000.

Wieland HA, Luddens H, and Seeburg PH. A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. *J Biol Chem* 267: 1426-1429, 1992.

Yee BK, Hauser J, Dolgov VV, Keist R, Mohler H, Rudolph U, and Feldon J. GABA receptors containing the α5 subunit mediate the trace effect in aversive and appetitive conditioning and extinction of conditioned fear. *Eur J Neurosci* 20: 1928-1936, 2004.

2. Interneurons in the hippocampal CA1 area exhibit region-specific tonic inhibition mostly mediated by α1-$GABA_A$ receptors

Edith M. Schneider Gasser, Jean-Marc Fritschy & Kaspar Vogt

Publication in preparation

Abstract

Using patch-clamp recordings of acute hippocampal slices, we show that, under intact GABA uptake, interneurons of the CA1 area differ markedly in the extent of their tonic inhibitory conductance. Tonic inhibition was consistently higher in stratum oriens than in stratum radiatum interneurons. In stratum lacunosum-moleculare, we identified a group of interneurons lacking tonic inhibition. As tonic conductance in hippocampal interneurons is diazepam-sensitive, we examined whether distinct $GABA_A$ receptor subtypes mediate these currents by using mice with a knock-in point mutation that renders individual $GABA_A$ receptor subtypes insensitive to this modulator. By comparing the effects of diazepam in interneurons from single, double and triple mutant mice, we could determine the contribution of α1, α2, α3 or α5-$GABA_A$ receptors to tonic inhibition. Triple-point mutated mice for the α1, α2 and α3 subunits, no increase in tonic inhibition after the application of diazepam occurred, whereas in double-mutant mice for the α2 and α3 subunits, tonic inhibition increased after diazepam application in 80% of stratum oriens and 70% of stratum radiatum interneurons. In α1 point-mutated mice, only 20% of the stratum oriens and 30% of the stratum radiatum interneurons exhibited increased tonic inhibition after diazepam application. Our results show that in the CA1 area of the hippocampus, tonic inhibition is mostly mediated by α1-$GABA_A$ receptors and in a subset of interneurons by α2-$GABA_A$ receptors. The higher amount of tonic inhibition in stratum oriens compared to stratum radiatum suggests regional differences in extracellular GABA concentration.

Introduction

$GABA_A$ receptors can be activated via a brief (phasic) exposure of postsynaptic receptors to a high concentration of GABA released from presynaptic vesicles, or by a continuous (tonic) exposure to ambient GABA binding to extrasynaptic receptors (Brickley et al., 1996; Nusser and Mody, 2002; Semyanov et al., 2004; Farrant and Nusser, 2005). Tonic activation of receptors depends on the ambient concentration of GABA in the extracellular space, which is controlled by a balance between GABA release and uptake. Tonic $GABA_A$-mediated inhibition regulates neuronal input resistance and, hence, excitability (Brickley et al., 2001; Semyanov et al., 2003), resulting in a decrease in the probability that a neuron reaches its threshold for firing an action potential.

Separate populations of $GABA_A$ receptors mediate the two forms of inhibitory conductance with different molecular assemblies and different affinities for GABA (Stell and Mody, 2002). $GABA_A$ receptors mediating phasic currents are typically composed of 2α/2β/γ subunits (Tretter et al., 1997; Barnard et al., 1998; Sieghart and Sperk, 2002). The γ2 subunit confers diazepam sensitivity to the receptors if expressed together with either α1, α2, α3 or α5 subunits (Mohler et al., 2002; Mohler, 2006), and is necessary for their aggregation at postsynaptic sites (Essrich et al., 1998; Nusser et al., 1998; Fritschy and Brunig, 2003).

Receptors containing the α4 or α6 subunit are frequently associated with the δ subunit and constitute major subtypes underlying tonic inhibition in dentate gyrus, ventrobasal complex of the thalamus, and cerebellar granule cell layer (Brickley et al., 2001; Belelli et al., 2002; Nusser and Mody, 2002; Stell et al., 2003; Jia et al., 2005; Belelli et al., 2006). These receptors are diazepam-insensitive, with high affinity for GABA, high sensitivity to neurosteroids and slow rates of desensitization (Brickley et al., 1999; Yeung et al., 2003; Belelli et al., 2006; Chandra et al., 2006). They do not form postsynaptic aggregates and are mainly located extrasynaptically. Finally, there are various types of $GABA_A$ receptors that mediate either tonic or phasic inhibition, depending on their cellular and subcellular location. In the CA1 area of the hippocampus, for example, diazepam-sensitive receptors are responsible for tonic inhibition under elevated ambient GABA concentrations, whereas at low concentrations, diazepam-insensitive, δ subunit-containing receptors are predominantly active (Semyanov et al., 2003; Caraiscos et al., 2004b; Scimemi et al., 2005). The former are $GABA_A$ receptors containing the α5 subunit that are localized in

pyramidal cells (Brunig et al., 2002; Crestani et al., 2002; Prenosil et al., 2006). In subiculum, expression of the α5 subunit is very low and tonic inhibition is likely mediated by α1-$GABA_A$ receptors (Prenosil et al., 2006). Hippocampal interneurons exhibit prominent tonic zolpidem-sensitive currents (Semyanov et al., 2003), but the $GABA_A$ receptor subtypes involved have not yet been identified. Therefore, the aim of the present study was to address this issue by analyzing the contribution to tonic inhibition of each α-subunit variant forming diazepam-sensitive $GABA_A$ receptors in interneurons.

Using *in vitro* patch-clamp electrophysiology, we recorded $GABA_A$ receptor-mediated currents in interneurons located in stratum oriens, radiatum and lacunosum-moleculare to determine whether tonic inhibition is layer- and/or cell type-specific. Interneurons were classified according to their spiking properties and were filled with biocytin for morphological characterization. The contribution of different diazepam-sensitive $GABA_A$ receptor subtypes to tonic inhibition was assessed using knock-in mice carrying an H101R point mutation in the α1, α2, α3 or α5 subunit that rendered the corresponding $GABA_A$ receptor insensitive to diazepam. The effect of diazepam on the holding current was compared between the different genotypes.

Methods

Mutant mice breeding

Experiments were performed in 129/SvJ knock-in mice carrying diazepam-insensitive $GABA_A$ receptor subtypes obtained by a histidine-to-arginine point-mutation in the α1, α2, α3 or α5 subunit gene [α1(H101R), α2(H101R) and α3(H126R), α5(H105R)] (Benson et al., 1998; Rudolph et al., 1999; Low et al., 2000) introduced by homologous recombination in embryonic stem cells. Mice carrying a point mutation in two (α12, α23) and even three (α123) α-subunit variants were obtained by intercrossing single mutant mice (Prenosil et al., 2006). All experiments were approved by the cantonal Veterinary Office of Zurich and were performed in accordance with the European Community Council Directive (86/609/EEC).

Slice preparation

Mice of both sexes (P18-24) were anaesthetized with inhaled isoflurane and decapitated. The brains were removed quickly and placed in ice-cold artificial cerebrospinal fluid (ACSF, composition in mM: NaCl 125, $NaHCO_3$ 26, NaH_2PO_4 1.25, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2.5, glucose 11, oxygenated with 95% O_2/5% CO_2). The brain was affixed to a vibratome stage (Microm HM 650 V, Microm International AG, Volketswil, Switzerland) with cyanoacrylate and kept in the ice-cold ACSF for slicing. Parasagittal 400 μm thick hippocampal slices were prepared and incubated at 33°C for 20 min, prior to being stored at room temperature (25°C) in oxygenated ACSF.

Electrophysiological recordings

Slices were visualized with a CCD camera (PCO Vx45; Till Photonics, Germany) mounted on an upright microscope (BX51WI; Olympus, Switzerland), equipped with a long working-distance water-immersion objective (Xlumplan FI 20X, 0.95 numerical aperture), a fourfold magnification changer, Nomarski-type differential interference contrast and infrared illumination. Patch electrodes were pulled from borosilicate glass (GC150TC; Clark Instruments, UK) and had an open tip resistance of 3-4 MΩ when filled with the internal solution.

Whole-cell voltage-clamp recordings in wildtype mice and different α-subunit mutants were made at room temperature (unless indicated) with a continuous perfusion (1 ml/min) of ACSF, at a holding potential of –60 mV with a high chloride-containing internal solution (composition in mM: CsCl 100, $MgCl_2$ 2, EGTA 1, MgATP 2, GTP 0.3, HEPES 40, pH 7,2, 300 mOsm) or at –50 mV with a low chloride-containing internal solution (composition in mM: KGlu 130, EGTA 1, HEPES 10, MgATP 5, NaGTP 0.5, NaCl 5, pH 7.3, 295 mOsm). Tonic inhibition was determined by the change in holding current after application of the $GABA_A$ receptor antagonist picrotoxin (2 or 100 μM, dissolved in DMSO). Bicuculline (10 μM) was also applied in some experiments to confirm $GABA_A$ receptor selectivity of the antagonists. Excitatory synaptic transmission was blocked with 2 mM kynurenic acid, and $GABA_B$ receptors were blocked with the antagonist CGP 55845 (1 μM, dissolved in DMSO). The sensitivity to diazepam (1 μM, dissolved in DMSO) was measured by applying the drug to the bath solution. In some experiments DMSO alone was applied to the bath solution to rule out any effect of the solvent on tonic inhibition.

Recorded interneurons were filled with biocytin 1% for later morphological identification. Recordings were made using a Multiclamp 700A patch-clamp amplifier (Axon Instruments, Inc., USA), low-pass filtered at 5 kHz, digitized at 20 kHz (PCI 6035; National Instruments, USA), stored and analyzed off-line using IGOR Pro software (Wave Metrics, Lake Oswego, USA). Access resistance was monitored throughout the experiments using a –5 mV voltage step every 10 seconds. The access was <20 MΩ, and results were discarded if they changed by more than 20% during the recording. The current decay after the voltage step was used to calculate the capacitance of the neurons.

Data analysis

Tonic current was analyzed with a custom-written macro running under IGOR Pro. Seal tests were deleted from the continuous recordings. Each 10-second baseline current period was represented as one point for all histogram plots. The average baseline holding current was calculated after intracellular chloride equilibration and 3-4 minutes after diazepam and picrotoxin application. The magnitude of the tonic current was obtained by subtracting the current recorded in the presence of picrotoxin or bicuculline from the baseline current. For each experiment, changes in holding current after drug application were statistically compared to baseline with paired Student's t-test. Differences between genotypes were analyzed with one-way ANOVA and Bonferroni's post-hoc multiple comparisons tests.

Morphological characterization

Patch pipettes were carefully removed from the recorded cells to avoid biocytin leakage and slices were fixed for 1 day in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB) (pH 7.3). Slices were then washed in 0.1 M PB and embedded overnight in 30% sucrose solution diluted in 0.1 M PB for cryopreservation. Slices were then frozen at –40°C in dimethylbutane and stored at –20°C until revelation Biocytin-filled cells were visualized by incubating the sections in avidin-biotin-horseradish peroxidase (HRP) complex (ABC 1:100 in PB 0.1 M) (Vectastain Elite Kite, Vector Laboratories, Burlingame, CA, USA) for 3 hours. To reveal the HRP staining, the reaction product was processed with diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO, USA) as chromogen. Sections were then mounted onto gelatin-coated slides, air-dried, dehydrated in ethanol, cleared in xylene, and coverslipped with Eukitt (Erne Chemie, Dällikon, Switzerland). Cells were examined with a light microscope (Nikon E-400)

equipped with a camera lucida and drawings of labeled interneurons lacking tonic inhibition were made from serial sections using a 20× (N.A. 0.75) objective.

Drugs

Chemicals were purchased from Sigma/Fluka (Switzerland) or Tocris Inc. (ANAWA Inc, Switzerland). Diazepam was provided by Hoffmann-La Roche Ltd (Switzerland). We tested DMSO alone at the appropriate concentrations on IPSCs to rule out any direct effects when it was used as a solvent.

Results

Tonic $GABA_A$ receptor-mediated inhibition in mouse hippocampal CA1 stratum oriens and stratum radiatum interneurons

We recorded spontaneous activity in whole-cell voltage-clamp mode from stratum oriens and stratum radiatum interneurons in wildtype mouse hippocampal slices. The internal solution contained a high cesium chloride concentration so that $GABA_A$ receptor-mediated currents were inward at –60 mV. $GABA_B$ receptors were blocked in all recordings. Application of 100 μM picrotoxin caused an outward drift in the holding current in all recorded interneurons from stratum oriens (e.g. Fig. 1A, n = 8) and stratum radiatum (e.g. Fig 1B, n = 10). eIPSCs and sIPSCs were completely abolished by picrotoxin as verified by extracellular stimulation (data not shown).

The effect of picrotoxin on the holding current was significantly different between stratum oriens and stratum radiatum interneurons. After application of picrotoxin, a double drift in the holding current was observed in all recorded stratum oriens interneurons (n = 21) compared to the drift in stratum radiatum interneurons (n = 25) (Fig. 1, Table 1). Application of a low concentration of picrotoxin (2 μM), which is reported to preferentially block GABA-bound $GABA_A$ receptors rather than unbound ones (Newland and Cull-Candy, 1992; Semyanov et al., 2003), produced a change in the holding current similar to that registered with high picrotoxin concentrations, but no significant reduction in the peak amplitude of the sIPSCs was observed (Prenosil et al., 2006). These results suggest the participation of $GABA_A$ receptors with different affinities for GABA in phasic and tonic forms of inhibition, as well as a higher affinity for GABA in tonically active $GABA_A$

receptors. Because picrotoxin also antagonizes glycine receptors, we applied another $GABA_A$ receptor antagonist, bicuculline (10 μM). Bicuculline similarly abolished sIPSCs and reduced the holding current as picrotoxin in stratum radiatum interneurons (n = 4) but to a lesser extent in stratum oriens interneurons (n = 5), indicating either a higher GABA concentration or the possible involvement of glycine-mediated tonic inhibition in the stratum oriens. Tonic inhibition was also present in interneurons recorded at physiological temperature (32°C), indicating that the results obtained at room temperature are not due to an impaired GABA uptake leading to more GABA accumulation. Application of the selective GABA uptake inhibitor NO711 (2 μM) at room temperature increased the amount of tonic current (Fig. 1D, n = 7), providing a second line of evidence for an unaltered uptake machinery**.** The total drift in holding current after application of different antagonists and the number of experiments per genotype are shown in Table 1.

TABLE 1. ***Effects of $GABA_A$ receptor antagonists on holding current in CA1 hippocampal interneurons***

	ΔI hold, pA (n)
Interneurons in stratum oriens	
Picrotoxin 2 μM (25°C)	30 ± 11 (4)
Picrotoxin 100 μM (25°C)	27 ± 5 (8)
Picrotoxin 2 μM (32°C)	34 ± 14 (5)
Picrotoxin 100 μM (32°C)	34 ± 10 (4)
Bicuculline 10 μM (25°C)	18 ± 6 (5)
Interneurons in stratum radiatum	
Picrotoxin 2 μM (25°C)	12 ± 5 (5)*
Picrotoxin 100 μM (25°C)	12 ± 4 (10)*
Picrotoxin 2 μM (32°C)	22 ± 3 (5)*
Picrotoxin 100 μM (32°C)	20 ± 4 (5)*
Bicuculline 10 μM (25°C)	14 ± 6 (4)*

* Significant ($P<0.05$) difference between stratum oriens and stratum radiatum interneurons (Student's t-test, see also graphs in figure 1).

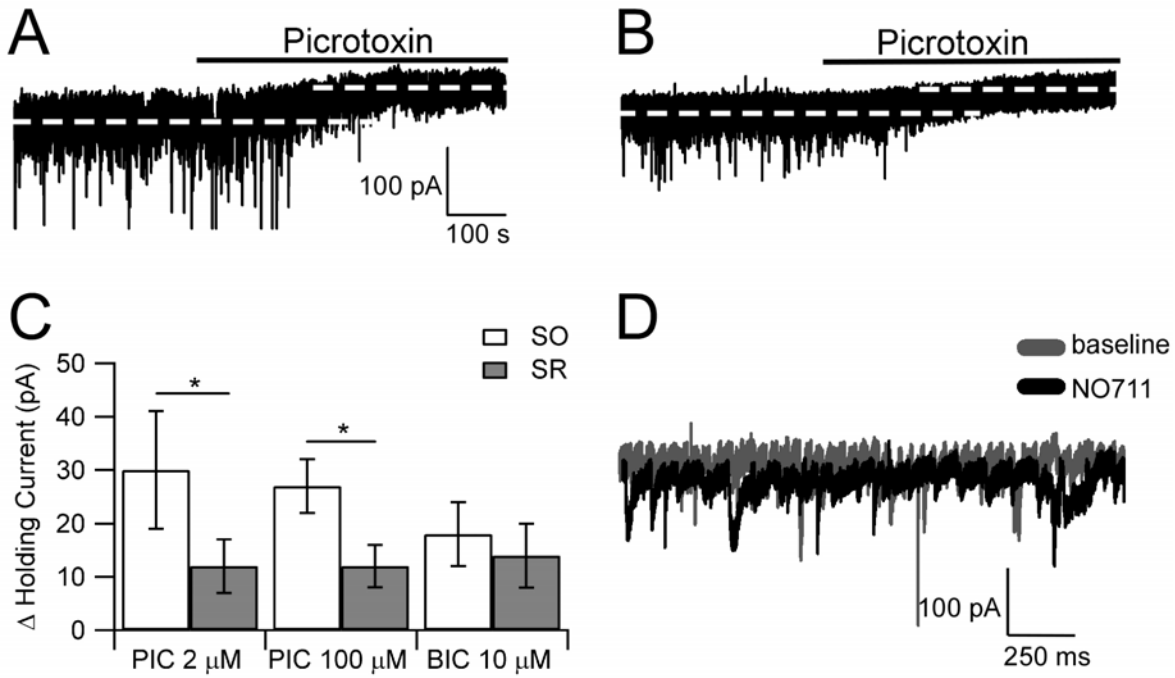


Figure 1. Interneurons in stratum oriens and radiatum express a tonic GABA$_A$ receptor mediated conductance being significantly higher in stratum oriens interneurons. *A, B:* Representative traces from stratum oriens (A) and stratum radiatum (B) interneurons showing the change in holding current after application of 100 μM picrotoxin. White dashed lines show the change in the holding current. Picrotoxin (100 μM) abolished spontaneous IPSCs. *C:* Absolute changes in holding current following application of the GABA$_A$ antagonist picrotoxin (PIC) in low or high concentrations (2 μM and 100 μM) and bicuculline (10 μM BIC). In stratum oriens interneurons bicuculline (10 μM) had a reduced effect compared to picrotoxin. *D:* Representative traces from stratum oriens interneuron showing the change in holding current after application of the GABA uptake blocker NO711 (2 μM). SO= stratum oriens, SR= stratum radiatum. Error bars (C) indicate standard deviation. *P < 0.05, Student's t-test. Numbers of experiments are shown in Table 1.

Different amounts of tonic current in stratum oriens and stratum radiatum interneurons suggest regional differences in extracellular GABA concentration

To determine the cause of the difference in tonic conductance between stratum oriens and stratum radiatum interneurons, we first determined whether interneurons in stratum oriens are larger than in stratum radiatum by measuring the capacitance of all recorded cells at room temperature. No correlation between capacitance and total leak current was observed in either layer (Fig. 2A). Moreover, although interneurons in stratum radiatum showed on average a significantly larger capacitance than interneurons in stratum oriens (Fig. 2B), their leak current was significantly smaller (Fig. 2C) and their input resistance slightly higher (Fig. 2D). As shown below, the characterization of the GABA$_A$ receptor subtypes

involved in tonic inhibition showed that α1-$GABA_A$ receptors are involved in most interneurons, thereby ruling out the possibility that the larger currents seen in stratum oriens are mediated by a different $GABA_A$ receptor subtype than in stratum radiatum. Therefore, extracellular GABA concentration may differ between the two layers in the CA1 area. The small leak current and large capacitance consistently recorded from different interneuron types in stratum radiatum further support the conclusion that the difference in tonic conductance results from a relatively low GABA content in this layer.

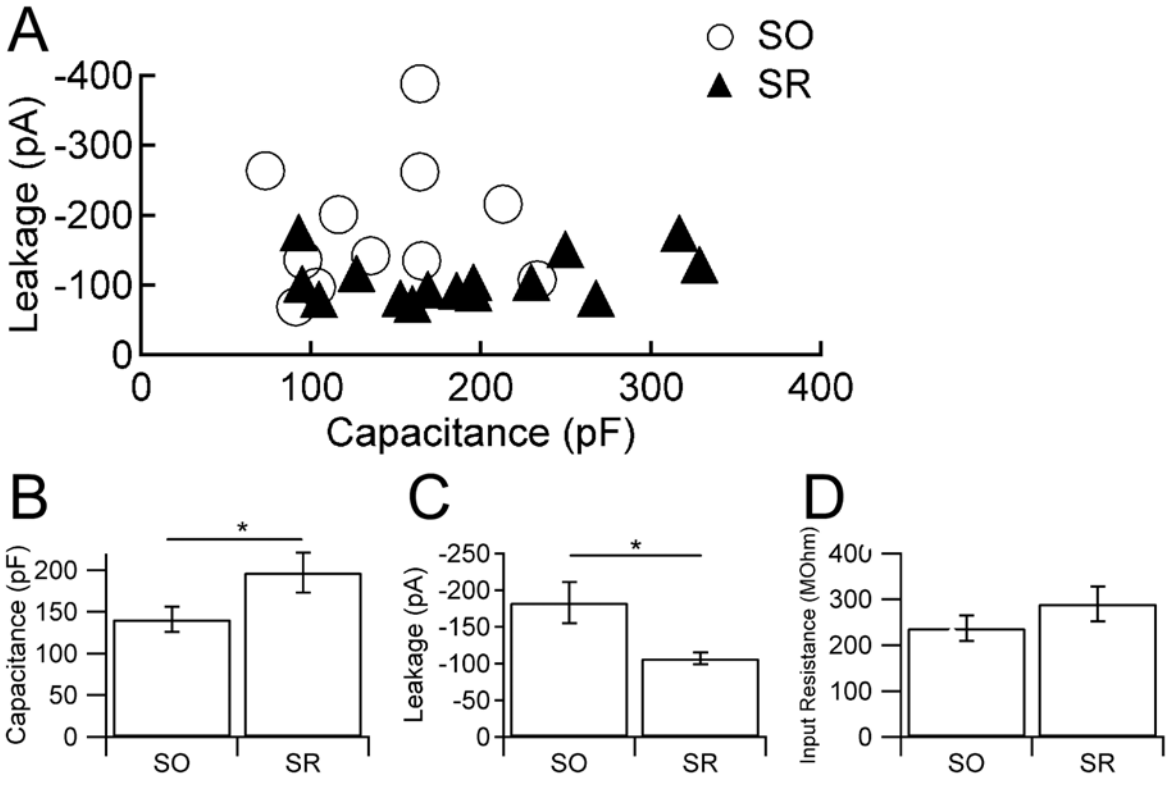


Figure 2. Difference in leakage between stratum oriens and stratum radiatum interneurons is not correlated with the capacitance of the cells. *A:* Correlation plot between capacitance and leakage shows no correlation between these parameters. Note that even cells with big capacitance are less leaky in stratum radiatum than in stratum oriens. *B:* Bar graphs representing average values from capacitance. *C:* Bar graphs representing average values from leakage. *D:* Bar graphs representing average values from input resistance in stratum oriens (SO) and stratum radiatum (SR) interneurons. n = 11 for SO and n = 16 for SR. Error bars B and C indicate standard error. *P < 0.05, Student's t-test.

Interneurons in stratum oriens and stratum radiatum with diverse firing patterns exhibit diazepam-sensitive $GABA_A$ receptor mediated tonic inhibition

Application of diazepam when recording from CA1 interneurons in wildtype mice increased the holding current in all interneurons from stratum oriens (n = 7) and stratum radiatum (n = 8) (Fig. 3A1-C1). This result confirms a previous report (Semyanov et al., 2003) that tonic inhibition in interneurons from the CA1 area is mediated by zolpidem-sensitive $GABA_A$ receptors in contrast to the diazepam-insensitive $GABA_A$ receptors seen in granule cells (Nusser et al., 1998). In order to classify the recorded interneurons, we determined their spiking patterns at the beginning of each experiment. Spike discharge was generated by 1-second 250 pA depolarizing pulses. Whole-cell current-clamp recordings with a low-chloride internal solution revealed three distinct patterns of inhibitory discharge: regular, irregular and clustered. Most interneurons in stratum oriens and stratum radiatum exhibited a regular firing pattern with weak accommodation (Fig. 3A and C, n = 13). Irregular spiking patterns were registered only in one stratum oriens and one stratum radiatum interneuron (Fig. 3B). Among the regular-spiking interneurons, five in stratum oriens and two in stratum radiatum showed fast-frequency (40-60 Hz) spiking patterns (Fig. 3A), whereas five interneurons in stratum radiatum and one in stratum oriens showed a spiking-pattern frequency between 10 and 20 Hz (Fig. 3C). The higher tonic conductance registered in stratum oriens could also be due to the type of interneuron present. Fast-frequency spiking interneurons are more common in stratum oriens and release more GABA, which could spill out of the synaptic cleft.

When threshold current injection (10-40 pA according to cell type) was applied in the presence of 2 μM picrotoxin, there was a marked increase in the number of action potentials were evoked in the interneurons (data not shown), confirming that blockade of tonic inhibition enhances their excitability.

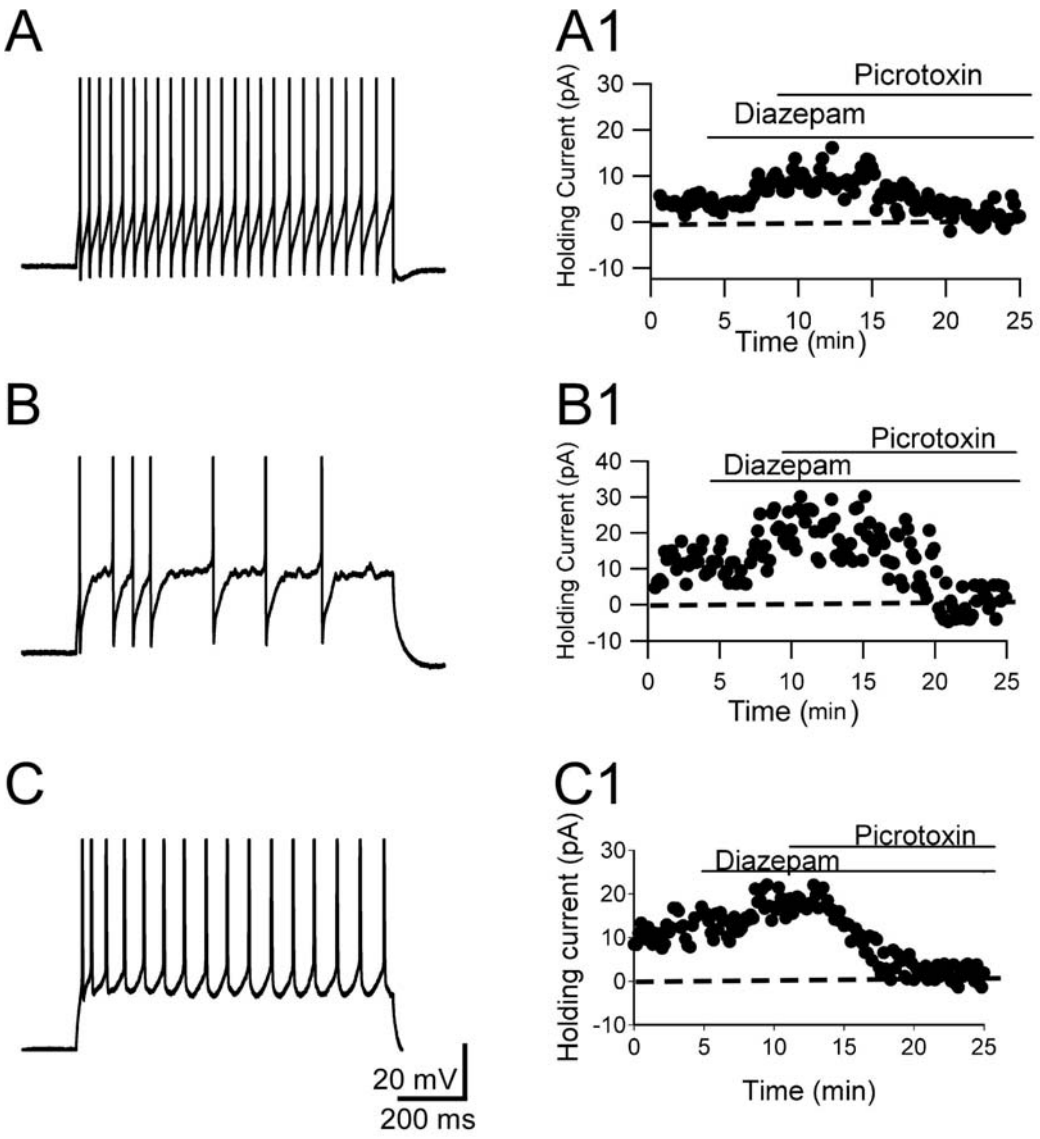


Figure 3. Spiking patterns from stratum oriens and stratum radiatum interneurons showing diazepam-sensitive tonic inhibition. A-C: Current-clamp traces showing the different spike trains after a one second 250 pA depolarizing current step. **A:** Fast spiking interneuron (30 – 60 Hz) (50%). **B:** Irregular spiking interneuron (10%). **C:** Regular spiking interneurons (10 - 20 Hz) with modest accommodation (40%). The percent values represent the frequency with which this sort of spiking pattern interneurons was registered. **A1-C1:** Change in holding current after the application of diazepam (outward drift) and picrotoxin (inward drift). Holding current values after picrotoxin application were aligned to 0 pA for easier comparison. All recorded interneurons had an increase in holding current after application of diazepam indicating the involvement of diazepam-sensitive $GABA_A$ receptors in tonic inhibition.

A subset of interneurons in stratum lacunosum-moleculare lack tonic inhibition

A subgroup of interneurons (n = 6) with the soma located at the border between stratum radiatum and stratum lacunosum-moleculare showed no drift in holding current after the application of either picrotoxin or bicuculline. Diazepam application also did not increase the holding current in these cells (Fig. 4C). Camera lucida reconstruction of biocytin-filled cells showed that they possess a characteristically round and small soma and aspiny dendrites (Fig. 4A1) oriented along the border of stratum lacunosum-moleculare and stratum radiatum (Fig. 4A). All recorded cells lacking tonic inhibition had the same location and morphology. The spiking patterns of these interneurons varied, showing either fast spiking (40-70 Hz), slightly accommodating action potentials (Fig. 4B1, n = 3) or clustered firing patterns (Fig. 4B2, n = 3). Unfortunately, further immunocytochemical characterization of these cells was not possible.

TABLE 2. ***Effect of 1 μM diazepam on holding current in different knock-in mice interneurons***

ΔI hold, pA (n)

Mice type	Interneurons in stratum oriens (SO)	Interneurons in stratum radiatum (SR)
Wild type	14 ± 4 (8)	10 ± 2 (10)
α5	11 ± 4 (5)	8 ± 3 (6)
α123	-3 ± 4 (5)*	0 ± 1 (6)*
α12	0 ± 3 (4)*	-2 ± 2 (7)*
α1	-2 ± 2 (5)*	2 ± 3 (7)*
α23	8 ± 4 (7)	4 ± 2 (11)

*Significant ($P<0.05$) difference between WT and knock-in values (Student's t-test, see also graphs in Figure 5).

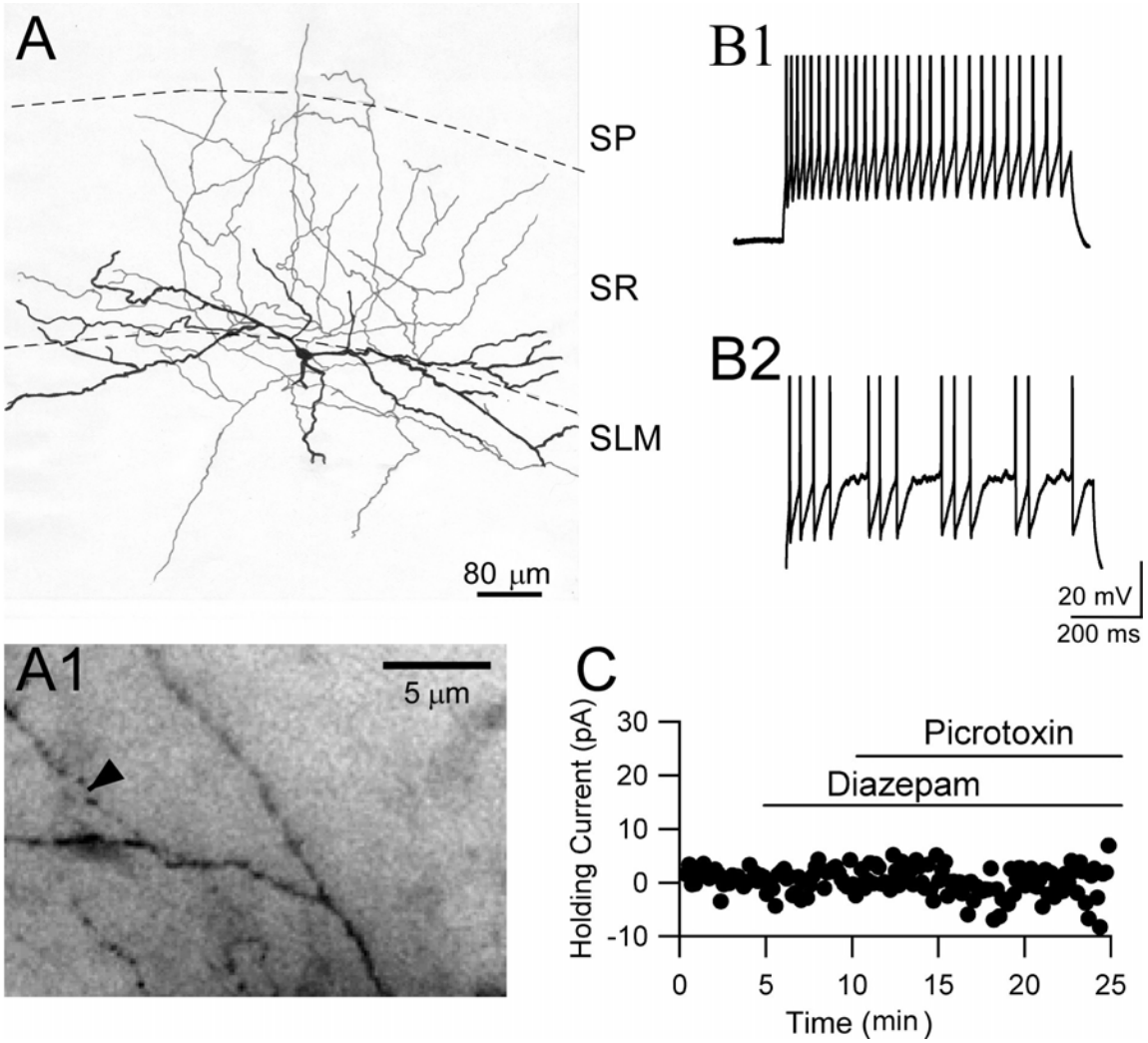


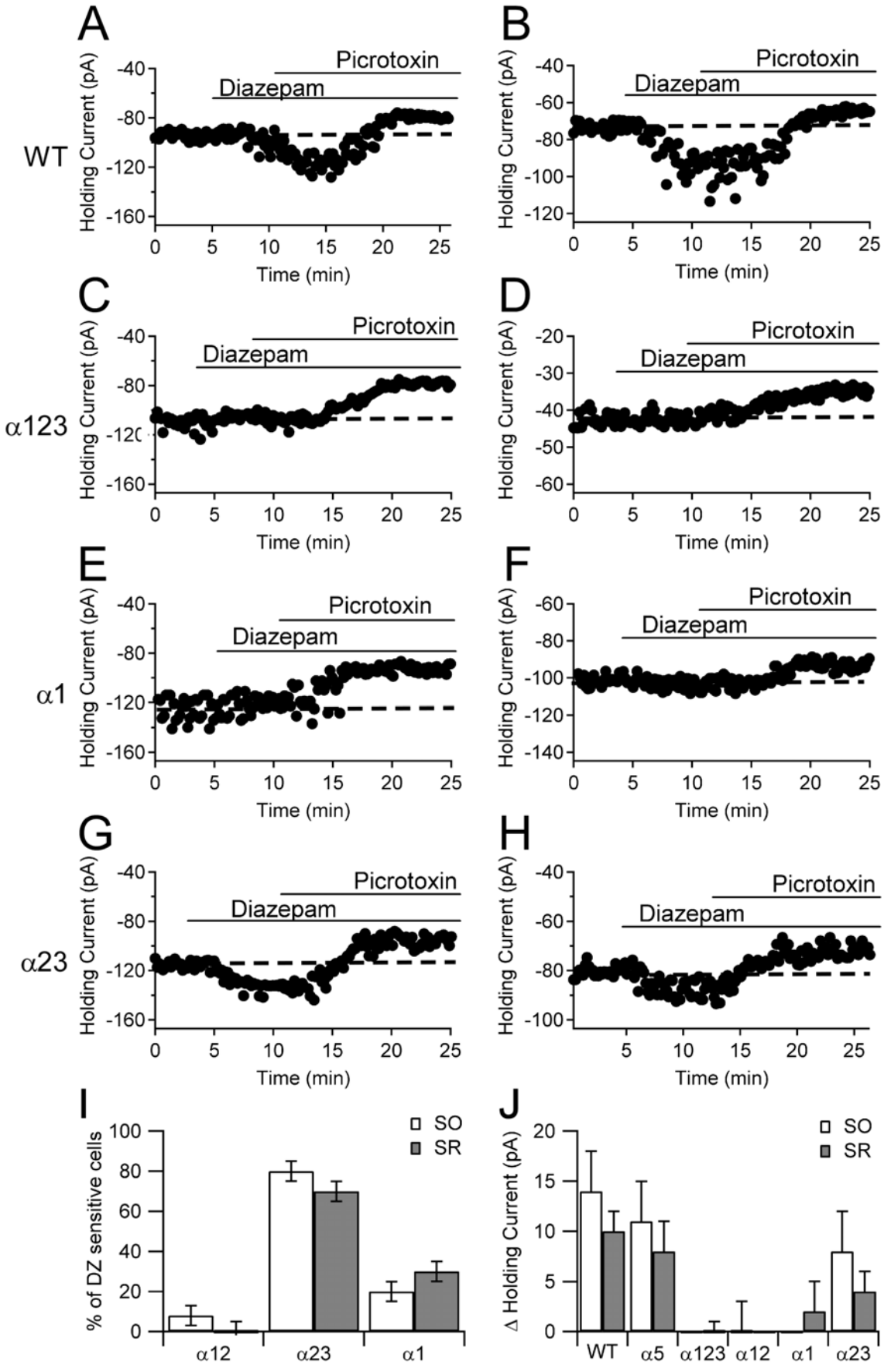
Figure 4. Spiking patterns of stratum lacunosum-moleculare interneurons not showing tonic inhibition. *A:* Camera lucida reconstruction of dendrites (thick line) and axonal (fine line) arborization of biocytin-filled CA1 interneuron with soma in the stratum lacunosum-moleculare. *A1:* Micrograph showing detail of dendrites and axonal (arrowhead) processed from the reconstructed cell. Axonal processed typically show varicosities. *B:* Current-clamp traces showing the spike trains after a one second 250 pA depolarizing current step. *B1*: Weak accommodating high frequency (40 - 70 Hz) spike train and *B2:* clustered spike trains are observed in these types of interneurons. *C*: Holding current shows no change after the application of diazepam and picrotoxin. SP = stratu pyramidale; SR = stratum radiatum; SLM = stratum lacunosum-moleculare.

Tonic inhibition in interneurons in stratum oriens and stratum radiatum is mostly mediated by α1 subunit-containing GABA$_A$ receptors

The increase in holding current after application of diazepam implies that tonic inhibition in CA1 interneurons is mediated, at least in part, by receptors containing the γ2 subunit and/or lacking the α4 subunit. In pyramidal cells, α5-GABA$_A$ receptors mediate tonic

inhibition (Caraiscos et al., 2004b; Prenosil et al., 2006) but in interneurons this subunit is normally absent (Brunig et al., 2002). We investigated the involvement of different diazepam-sensitive $GABA_A$ receptor subtypes that mediate tonic inhibition in interneurons by whole-cell voltage-clamp recordings from knock-in mice carrying diazepam-insensitive $GABA_A$ receptor subtypes. Figure 5 shows the drift in the holding current after the application of diazepam and picrotoxin in wildtype mice (Fig. 5A-B); α123-mice, in which only α5-$GABA_A$ receptors remain diazepam-sensitive (Fig. 5C-D); α1-mice, in which α5-, α3- and α2-$GABA_A$ receptors remain diazepam-sensitive (Fig. 5E-F) and α23-mice in which α1- and α5-$GABA_A$ receptors remain diazepam-sensitive (Fig. 5G-H). Recordings in α5- and α12- mice were also made (not shown). The average change in holding current after application of 1 μM diazepam to the various genotypes, and the number of recorded cells, is summarized in Table 2. In α123-mice, the effect of diazepam was completely abolished, confirming the absence of α5-$GABA_A$ receptors in interneurons (Fig. 5C-D). In α12-mice, only one cell out of twelve with the soma located close to the alveus showed a slight change in holding current after diazepam application, consistent with the presence of a specific population of interneurons carrying α3-$GABA_A$ receptors at this location and in the hilus (Brünig et al., 2002; Schneider Gasser et al., 2007).

Figure 5. Holding current changes in stratum oriens and stratum radiatum interneurons from wildtype and different α-subunit knock-in mice after the application of diazepam and picrotoxin. Left graphs (*A, C, E, G*) represent examples of stratum oriens interneurons and right graphs (*B, D, F, H*) of stratum radiatum interneurons. *A, B:* Change in holding current in wildtype (WT) mice. *C, D:* Change in holding current in α1, α2 and α3 knock-in mice (α123) showing no activation of α5-containing receptors in tonic inhibition. *E, F:* Change in holding current in α1 knock-in mice (α1) showing no activation of α2, α3 and α5-containing receptors in tonic inhibition. *G, H:* Change in holding current in α2 and α3 knock-in mice (α23) showing the involvement of α1-$GABA_A$ receptors in tonic inhibition. *I:* Percentage of interneurons in stratum oriens (SO) and radiatum (SR) showing increase in holding current after the application of diazepam in α12, α23 and α1 knock-in mice. Error bars represent S.E. *J:* Summary data plotted from average values of each recorded genotype showing the increase in holding current after application of diazepam. α5 = α5 knock-in mice and α12 = α1 and α2 knock-in mice. See also table 2. Error bars represent SD.


A
B
C
D
E
F
G
H
I
J
WT
α123
α1
α23
Picrotoxin
Diazepam
Holding Current (pA)
Time (min)
% of DZ sensitive cells
SO
SR
α12
α23
α1
Δ Holding Current (pA)
WT
α5
α123
α12
α1
α23

In α1-mice, only 20% of interneurons in stratum oriens and 30% in stratum radiatum were sensitive to diazepam (Fig. 5E-F), suggesting that α2-$GABA_A$ receptors contribute to tonic inhibition in a subgroup of interneurons. In contrast, in α23-mice, diazepam increased holding currents in 80% of stratum oriens and 70% of stratum radiatum interneurons, confirming the predominant role of α1-$GABA_A$ receptors in tonic inhibition in CA1 interneurons.

Discussion

Our study reveals layer-specific differences in the amplitude of tonic inhibition mediated by CA1 interneurons. In most cells, these currents are carried by α1-$GABA_A$ receptors, although a contribution of α2- and α3-$GABA_A$ receptors was also found in a minority of interneurons. A specific cell type identified in stratum lacunosum-moleculare was devoid of tonic inhibition. Altogether, these findings suggest that the density of extrasynaptic $GABA_A$ receptors and/or extracellular GABA concentrations may differ according to strata and cell type to control neuronal and network excitability.

The present data show that tonic $GABA_A$ receptor-mediated inhibition is present in CA1 interneurons from mice recorded in acute slices in the absence of GABA uptake inhibitors. The amplitude of tonic inhibition was similar at room and at physiological temperature, indicating that the uptake machinery is functional under both conditions. Application of the selective GABA-uptake inhibitor NO711 at room temperature increased the amount of tonic current, suggesting that the measured tonic current does not result from an altered GABA uptake. Unexpectedly, we observed that tonic conductance is mostly a function of the laminar position of the interneurons, rather than the physiological properties used here to define separate cell types. Interneurons in stratum oriens have a significantly and consistently larger tonic conductance than interneurons in stratum radiatum/lacunosum-moleculare independent of the type of $GABA_A$ receptor involved in mediating the tonic conductance.

Differences in extracellular GABA content according to strata may exist in the CA1 area. Engel et al. (1998) reported a laminar difference in GABA uptake and GABA transporter (GAT-1) expression in the rat CA1 area, and interneurons in stratum oriens were shown to

express less GAT-1 than interneurons in stratum radiatum/lacunosum-moleculare, so the regulation of GABA uptake seems to differ between layers, and most probably the lower expression of GAT-1 indicates a higher accumulation of extracellular GABA and therefore a higher tonic conductance. These results are also supported by the fact that tiagabine, a GABA-uptake blocker specific for the neuronal transporter GAT-1 (Borden et al., 1994), caused a marked prolongation of IPSCs that was significantly larger in stratum radiatum than in stratum oriens (Engel et al., 1998). Transporters can also undergo rapid redistribution between surface and intracellular compartments, and their function can be altered by phosphorylation (Quick et al., 2004) or intermolecular interactions (Hansra et al., 2004). So even when GABA release is unchanged, ambient GABA concentration, and thereby tonic inhibition, can be modulated by changes in uptake. Local GABA concentrations cannot be estimated, since they are also dependent on local differences in GABA release.

The firing patterns of hippocampal interneurons did not show a correlation with the amount of tonic conductance. Cells with the same spiking pattern in stratum oriens and stratum radiatum exhibited different amounts of tonic inhibition according to their location, whereas cells with different spiking patterns in the same layer had similar tonic activation. Therefore, the difference in tonic inhibition depends primarily on the location rather than on the subtype of interneuron. In stratum lacunosum-moleculare some cells exhibit tonic inhibition of the same amplitude as in stratum radiatum, which might reflect a lower extracellular GABA concentration compared to stratum oriens. Remarkably, a specific group of cells located along the border of the stratum radiatum and stratum lacunosum-moleculare with two types of spiking patterns and a similar dendritic morphology, lack tonic inhibition. The stratum lacunosum-moleculare is the main site of termination of axons from layer III of the entorhinal cortex. These axons form a direct excitatory pathway onto CA1 pyramidal cells (Gloveli et al., 1997), but also produce inhibition of pyramidal cells through activation of *feedforward* GABAergic interneurons whose dendrites are located in the stratum lacunosum-moleculare (Remondes and Schuman, 2002). It is quite possible that the interneurons involved in feedforward inhibition of CA1 pyramidal neurons are those described here as being devoid of tonic inhibition.

Low and high picrotoxin concentrations were shown to have the same effect on holding currents in our recordings, whereas sIPSCs were only abolished with high concentrations. The difference in blockade of tonic and phasic receptors by low picrotoxin concentration

does not imply differences in affinity of the receptors for picrotoxin, but implies a difference in affinity of the receptors for GABA, and therefore a difference in their activation. Thus, extrasynaptic $GABA_A$ receptors, which have a higher affinity for GABA, are blocked early, and by a low dose of picrotoxin (Prenosil et al., 2006). Bicuculline abolished sIPSCs in a similar way to picrotoxin but had a smaller effect on tonic inhibition in stratum oriens interneurons. The proportion of tonic current not blocked by bicuculline could be attributed to the fact that, as a competitive antagonist, it may be displaced from high GABA-affinity receptors when GABA concentrations rise. Other possible causes could be the presence of bicuculline-insensitive $GABA_C$ receptors, which are mostly extrasynaptically located and activated by spillover of synaptically released GABA (Alakuijala et al., 2006), or the presence of glycinergic tonic inhibition.

Differences in subunit composition between synaptic and extra- or perisynaptic receptors are reflected in the differential modulation of phasic and tonic inhibition by benzodiazepine site ligands. In dentate gyrus, tonic conductance is mediated by diazepam-insensitive $\alpha_4\beta\delta$ receptors (Nusser and Mody, 2002) and in cerebellar granule cells by diazepam-insensitive $\alpha_6\beta\delta$ receptors (Hamann et al., 2002), whereas in hippocampal pyramidal cells, tonic activation is mediated by $\alpha_5\beta_3\gamma_2$ receptors (Caraiscos et al., 2004a; Prenosil et al., 2006). In contrast, we show here that the tonic conductance in CA1 interneurons is mediated mainly by α1-$GABA_A$ receptors. It should be noted that enhanced tonic conductance in the presence of a positive allosteric modulator is not as straightforward to interpret as the lack of an effect, because any drug-induced increase in receptor affinity might recruit additional receptor populations (including synaptic receptors) that are not ordinarily activated by the low ambient GABA concentration.

Our study reveals layer-specific differences in the amplitude of tonic inhibition mediated by CA1 interneurons. In most cells, these currents are carried by α1-$GABA_A$ receptors, although a contribution of α2- and α3-$GABA_A$ receptors was also found in a minority of interneurons. A specific cell type identified in stratum lacunosum-moleculare was devoid of tonic inhibition. Altogether, these findings suggest that the density of extrasynaptic $GABA_A$ receptors and/or extracellular GABA concentrations may differ according to strata and cell type to control neuronal and network excitability**.**

References

Alakuijala A, Alakuijala J, Pasternack M (2006) Evidence for a functional role of GABA receptors in the rat mature hippocampus. Eur J Neurosci 23:514-520.

Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN, Langer SZ (1998) International Union of Pharmacology. XV. Subtypes of $GABA_A$ receptors: classification on the basis of subunit structure and receptor function. Pharmacol Rev 50:291-313.

Belelli D, Casula A, Ling A, Lambert JJ (2002) The influence of subunit composition on the interaction of neurosteroids with $GABA_A$ receptors. Neuropharmacology 43:651-661.

Belelli D, Herd MB, Mitchell EA, Peden DR, Vardy AW, Gentet L, Lambert JJ (2006) Neuroactive steroids and inhibitory neurotransmission: mechanisms of action and physiological relevance. Neuroscience 138:821-829.

Benson JA, Low K, Keist R, Mohler H, Rudolph U (1998) Pharmacology of recombinant $GABA_A$ receptors rendered diazepam-insensitive by point-mutated α-subunits. FEBS Lett 431:400-404.

Borden LA, Murali Dhar TG, Smith KE, Weinshank RL, Branchek TA, Gluchowski C (1994) Tiagabine, SK&F 89976-A, CI-966, and NNC-711 are selective for the cloned GABA transporter GAT-1. Eur J Pharmacol 269:219-224.

Brickley SG, Cull-Candy SG, Farrant M (1996) Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of $GABA_A$ receptors. J Physiol 497 (Pt 3):753-759.

Brickley SG, Cull-Candy SG, Farrant M (1999) Single-channel properties of synaptic and extrasynaptic $GABA_A$ receptors suggest differential targeting of receptor subtypes. J Neurosci 19:2960-2973.

Brickley SG, Revilla V, Cull-Candy SG, Wisden W, Farrant M (2001) Adaptive regulation of neuronal excitability by a voltage-independent potassium conductance. Nature 409:88-92.

Brunig I, Scotti E, Sidler C, Fritschy JM (2002) Intact sorting, targeting, and clustering of $GABA_A$ receptor subtypes in hippocampal neurons *in vitro*. J Comp Neurol 443:43-55.

Caraiscos VB, Newell JG, You-Ten KE, Elliott EM, Rosahl TW, Wafford KA, MacDonald JF, Orser BA (2004a) Selective enhancement of tonic GABAergic inhibition in murine

hippocampal neurons by low concentrations of the volatile anesthetic isoflurane. J Neurosci 24:8454-8458.

Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF, Orser BA (2004b) Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing $GABA_A$ receptors. Proc Natl Acad Sci U S A 101:3662-3667.

Chandra D, Jia F, Liang J, Peng Z, Suryanarayanan A, Werner DF, Spigelman I, Houser CR, Olsen RW, Harrison NL, Homanics GE (2006) $GABA_A$ receptor α4 subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. Proc Natl Acad Sci U S A 103:15230-15235.

Crestani F, Keist R, Fritschy JM, Benke D, Vogt K, Prut L, Bluthmann H, Mohler H, Rudolph U (2002) Trace fear conditioning involves hippocampal α5-$GABA_A$ receptors. Proc Natl Acad Sci U S A 99:8980-8985.

Engel D, Schmitz D, Gloveli T, Frahm C, Heinemann U, Draguhn A (1998) Laminar difference in GABA uptake and GAT-1 expression in rat CA1. J Physiol 512 (Pt 3):643-649.

Essrich C, Lorez M, Benson JA, Fritschy JM, Luscher B (1998) Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the γ2 subunit and gephyrin. Nat Neurosci 1:563-571.

Farrant M, Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. Nat Rev Neurosci 6:215-229.

Fritschy JM, Brunig I (2003) Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications. Pharmacol Ther 98:299-323.

Gloveli T, Schmitz D, Empson RM, Dugladze T, Heinemann U (1997) Morphological and electrophysiological characterization of layer III cells of the medial entorhinal cortex of the rat. Neuroscience 77:629-648.

Hamann M, Rossi DJ, Attwell D (2002) Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex. Neuron 33:625-633.

Hansra N, Arya S, Quick MW (2004) Intracellular domains of a rat brain GABA transporter that govern transport. J Neurosci 24:4082-4087.

Jia F, Pignataro L, Schofield CM, Yue M, Harrison NL, Goldstein PA (2005) An extrasynaptic $GABA_A$ receptor mediates tonic inhibition in thalamic VB neurons. J Neurophysiol 94:4491-4501.

Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, Fritschy JM, Rulicke T, Bluethmann H, Mohler H, Rudolph U (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. Science 290:131-134.

Mohler H (2006) $GABA_A$ receptor diversity and pharmacology. Cell Tissue Res 326:505-516.

Mohler H, Fritschy JM, Rudolph U (2002) A new benzodiazepine pharmacology. J Pharmacol Exp Ther 300:2-8.

Newland CF, Cull-Candy SG (1992) On the mechanism of action of picrotoxin on GABA receptor channels in dissociated sympathetic neurones of the rat. J Physiol 447:191-213.

Nusser Z, Mody I (2002) Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. J Neurophysiol 87:2624-2628.

Nusser Z, Sieghart W, Somogyi P (1998) Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. J Neurosci 18:1693-1703.

Prenosil GA, Schneider Gasser EM, Rudolph U, Keist R, Fritschy JM, Vogt KE (2006) Specific subtypes of $GABA_A$ receptors mediate phasic and tonic forms of inhibition in hippocampal pyramidal neurons. J Neurophysiol 96:846-857.

Quick MW, Hu J, Wang D, Zhang HY (2004) Regulation of a GABA transporter by reciprocal tyrosine and serine phosphorylation. J Biol Chem 279:15961-15967.

Remondes M, Schuman EM (2002) Direct cortical input modulates plasticity and spiking in CA1 pyramidal neurons. Nature 416:736-740.

Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Mohler H (1999) Benzodiazepine actions mediated by specific $GABA_A$ receptor subtypes. Nature 401:796-800.

Scimemi A, Semyanov A, Sperk G, Kullmann DM, Walker MC (2005) Multiple and plastic receptors mediate tonic $GABA_A$ receptor currents in the hippocampus. J Neurosci 25:10016-10024.

Semyanov A, Walker MC, Kullmann DM (2003) GABA uptake regulates cortical excitability via cell type-specific tonic inhibition. Nat Neurosci 6:484-490.

Semyanov A, Walker MC, Kullmann DM, Silver RA (2004) Tonically active $GABA_A$ receptors: modulating gain and maintaining the tone. Trends Neurosci 27:262-269.

Sieghart W, Sperk G (2002) Subunit composition, distribution and function of $GABA_A$ receptor subtypes. Curr Top Med Chem 2:795-816.

Stell BM, Mody I (2002) Receptors with different affinities mediate phasic and tonic $GABA_A$ conductances in hippocampal neurons. J Neurosci 22:RC223.

Stell BM, Brickley SG, Tang CY, Farrant M, Mody I (2003) Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subunit-containing $GABA_A$ receptors. Proc Natl Acad Sci U S A 100:14439-14444.

Tretter V, Ehya N, Fuchs K, Sieghart W (1997) Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. J Neurosci 17:2728-2737.

Yeung JY, Canning KJ, Zhu G, Pennefather P, MacDonald JF, Orser BA (2003) Tonically activated $GABA_A$ receptors in hippocampal neurons are high-affinity, low-conductance sensors for extracellular GABA. Mol Pharmacol 63:2-8.

3. Immunofluorescence in brain sections: simultaneous detection of presynaptic and postsynaptic proteins in identified neurons

Edith M. Schneider Gasser[1], Carolin J. Straub[1], Patrizia Panzanelli[2], Oliver Weinmann[3], Marco Sassoè-Pognetto[2,4], Jean-Marc Fritschy

[1] Equal contribution

[2]Department of Anatomy, Pharmacology, and Forensic Medicine, University of Turin, Corso Massimo d'Azeglio 52, I – 10126 Torino, Italy

[3] Brain Research Institute, University of Zurich, Winterthurerstrasse 190, CH – 8057 Zurich, Switzerland

[4] Rita Levi Montalcini Center for Brain Repair, University of Turin, Italy

Published in: Nature Protocols 1: 1887 - 1897, 2006

Abstract

Elucidating the molecular organization of synapses is essential for understanding brain function and plasticity. Immunofluorescence, combined with various fluorescent probes, is a sensitive and versatile method for morphological studies. However, analysis of synaptic proteins in situ is limited by epitope masking upon tissue fixation. Furthermore, postsynaptic proteins (ionotropic receptors, scaffolding proteins) often require weaker fixation for optimal detection than most cell type and presynaptic markers, thereby hindering simultaneous visualization of these molecules. Here, we present an alternative to perfusion-fixation overcoming these restrictions. Brief tissue fixation shortly after interruption of vital functions preserves both morphology and antigenicity. Using markers of inhibitory synapses in rodent brain, selective detection of $GABA_A$ receptors and the scaffolding protein gephyrin in relation to identified presynaptic terminals is feasible by confocal laser scanning microscopy in neurochemically defined neurons. The most sophisticated of these protocols can be combined with electrophysiology for correlative morphofunctional studies of synapses in CNS. Tissue preparation and immunofluorescence staining require 2-3 consecutive days for completion.

Introduction

The function of synapses crucially depends on proper alignment of pre- and postsynaptic specializations and on targeting and anchoring of the neurotransmitter release machinery and postsynaptic density proteins to appropriate sites. Immunoelectron microscopy studies have demonstrated the existence of membrane microdomains, either pre- or postsynaptically, in which specific molecules are clustered with high density (e.g., [1-9]; reviewed in [10]). While allowing determination of the precise ultrastructural localization of antigen(s) of interest, immunoelectron microscopy typically is limited to a small sample and cannot readily be combined with complementary functional analyses in living tissue. Furthermore, some antigens are washed out during dehydration and become undetectable upon conventional tissue preparation for electron microscopy. Added sensitivity can be achieved by plunge freezing and low temperature Lowicryl embedding [11, 12] or freeze-fracture replica labeling [13-16]. However, these methods are not routinely established in most laboratories and are technically challenging.

Although ultrastructural analysis is a prerequisite for studying the morphology of synapses, the resolution of epifluorescence, in particular when combined with confocal laser scanning microscopy, is sufficient for clearly distinguishing pre- and postsynaptic compartments in tissue sections [17, 18]. Furthermore, a vast array of fluorescent probes can be investigated in combination with immunofluorescence. Therefore, this approach is in principle suitable to analyze the distribution of synaptic proteins in CNS tissue with the added advantages that several molecules can be co-visualized using multiple fluorescence labeling.

Histology and immunohistochemistry, in particular, require appropriate fixation for the morphological preservation of the structures to be analyzed. Current chemical fixatives include aldehydes or organic solvents, such as methanol or acetone. Aldehyde fixation is a prerequisite for immunohistochemical detection of soluble proteins, including neurotransmitters, neuropeptides, calcium-binding proteins, and reporter proteins such as GFP, to avoid diffusion out of damaged cells during the staining procedure. However, loss of antigenicity and/or epitope masking due to tissue fixation is a major limitation of immunohistochemistry, notably for the detection of postsynaptic proteins [19-21]. Aldehyde fixation may alter antigenic sites by cross-linking tissue proteins and appears to limit the penetration of antibodies into the postsynaptic density and into the synaptic cleft, a narrow

space separating the pre- and postsynaptic elements and filled with extracellular matrix molecules. Accordingly, classical fixation of brain tissue by intravascular perfusion with an aldehyde solution (or merely by immersion in the fixative) leads to a reduced detection of postsynaptic proteins. In contrast, immunohistochemical staining of vesicle-associated presynaptic proteins, such as vesicular transporters, usually requires excellent tissue preservation, hence a strong fixation, and therefore the simultaneous detection of pre- and postsynaptic markers by immunofluorescence is challenging [22]. Accordingly, there are only a few studies in which satisfactory detection of cell type markers or neutrotransmitters was combined successfully with immunofluorescence of postsynaptic proteins. While it appears of prime importance to understand the chemical neuroanatomy of neurons and synapses, these technical limitations have greatly hampered progress in this field.

A number of solutions have been proposed to resolve this issue, including various antigen-retrieval procedures [19], digestion of the fixed tissue with proteolytic enzymes [23, 24], inhibition of proteolysis during fixation [22], and short-fixation techniques to achieve a compromise between labeling efficiency and morphological preservation [20]. In our studies, we have focused on the latter approach, attempting to reconcile the opposite fixation requirements of pre- and postsynaptic proteins for optimal immunohistochemical detection. By rapidly freezing tissue prior to immersion-fixation or by briefly fixing tissue slices as soon as possible after interruption of vital functions, it is possible to avoid loss of soluble proteins by proteolysis or diffusion. Our experience is mainly derived from the study of inhibitory synapses. Such synapses are characterized by $GABA_A$ and/or glycine receptors, which are heterooligomeric ligand-gated channels assembled from a large family of subunits [25-28]. These receptors are clustered at postsynaptic sites with gephyrin [29], a scaffolding molecule which is selectively located in all but a few types of inhibitory synapses [30], and therefore is a reliable marker of such synapses. Our specific aim is to correlate expression of specific neuronal markers in defined synaptic circuits with distinct $GABA_A$ (or glycine) receptor subtypes, distinguished by their subunit composition.

Here, we describe a protocol allowing the detection of postsynaptic clusters of $GABA_A$ receptors and gephyrin by immunofluorescence. Three alternative methods of tissue preparation are presented (Fig. 1), in order to visualize these postsynaptic proteins together with markers of identified cell types or presynaptic terminals. These procedures use freshly resected brain tissue rather than perfusion-fixed tissue. They have been tested extensively

in mouse and rat brain tissue (see Table 2) but should be suitable for other small vertebrates with minimal adjustments.

- Option 1A (Fig. 1) allows detection of $GABA_A$ receptors and gephyrin with the highest efficiency. Staining of presynaptic vesicles (e.g., vesicular transporters) and cytoplasmic proteins is rather poor; likewise, staining of juvenile tissue is limited due to a very weak fixation. However, this procedure is fast and allows cutting entire brain sections for rapid screening of regional distribution patterns.
- Option 1B (Fig. 1) provides a good compromise between sensitivity and morphological preservation. In addition to $GABA_A$ receptor subunits and gephyrin, it also allows visualization of cytoplasmic cell markers in the soma and dendrites and of some presynaptic molecules, notably those associated with presynaptic vesicles. Detection of GFP transgenically expressed as reporter protein is poor. This protocol is best suited for brain regions that can readily be isolated (e.g., olfactory bulb) and that usually are not adequately preserved with option 1A (retina; juvenile tissue).
- Option 1C (Fig. 1) provides, in addition to option 1B, a better preservation of cell morphology and detection of soluble cell markers, including GFP, neurotransmitters, calcium binding proteins, etc. Here, brain slices are prepared first using the same protocol as for electrophysiological recordings; therefore, both methods can be combined on the same animal (or even the same slices). Another advantage of option 1C is that selected slices can be processed further for electron microscopy. It is the most time-consuming of the three options and does not allow parallel processing of multiple animals.

As a word of caution, it should be emphasized that this protocol is not aimed at replacing perfusion-fixation, which is a convenient and reliable method for adequate tissue preservation and suitable for most Immunohistochemical applications. However, since epitope-masking of postsynaptic proteins often occurs in perfusion-fixed tissue, the protocols presented here provide a way to circumvent this limitation for studies of the subcellular distribution of synaptic proteins. Furthermore, by avoiding perfusion-fixation, this protocol can be combined with functional or biochemical analyses performed on the same animals.

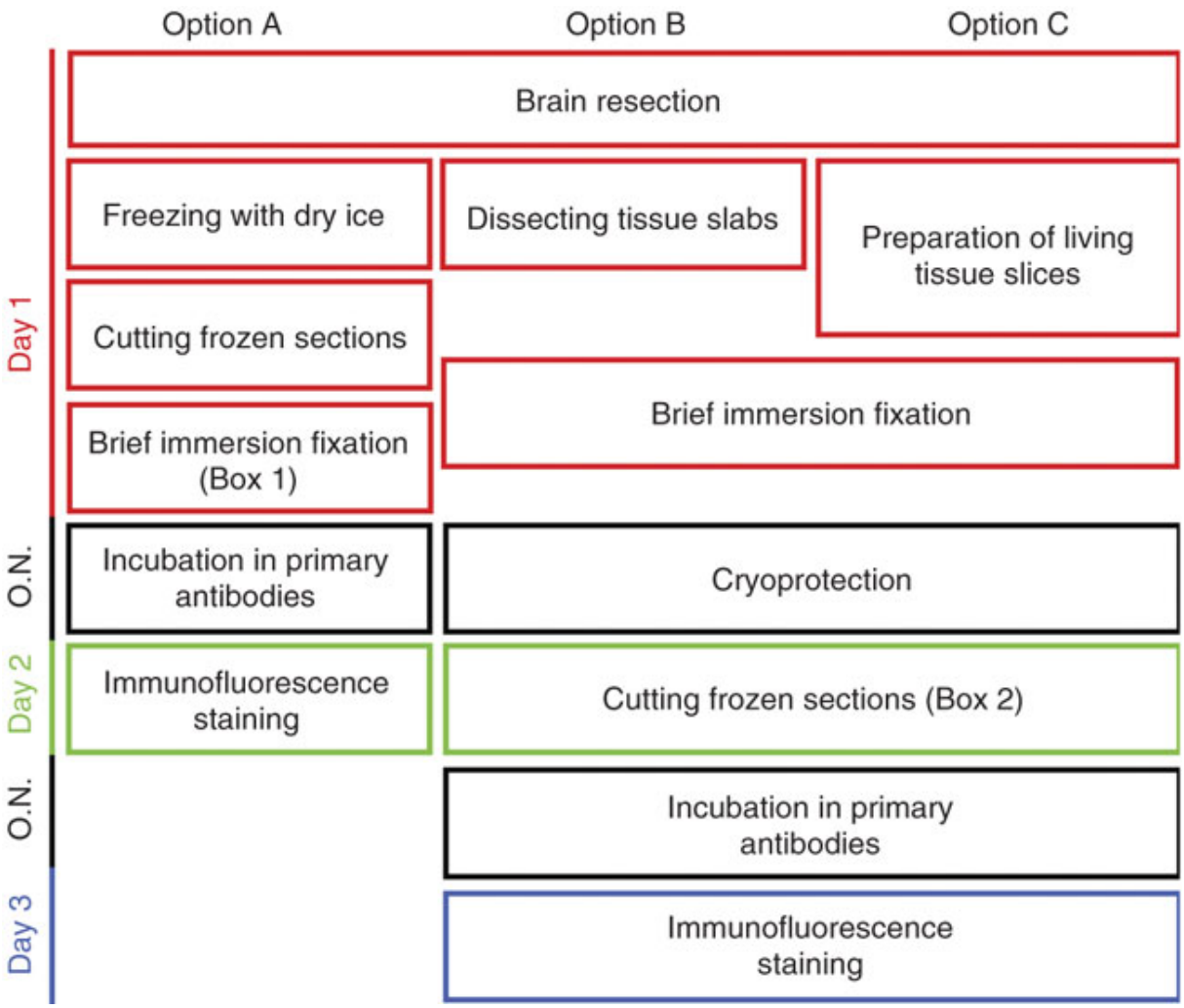


Figure 1: A flow chart of the experimental procedure for the three options of tissue preparation. Each day is indicated by a distinct color, with overnight (O.N.) incubation being shown in black.

Materials

REAGENTS

- Volatile anesthetic (e.g., isoflurane, Abbott; Methophan®, Janssen GmbH)
  ! CAUTION Harmful if inhalated. Work in a ventilated hood.
- Powdered dry ice.
- NaOH (Fluka, Sigma) **!** CAUTION Corrosive. Wear gloves and work in a ventilated hood.
- Sodium phosphate buffer (Fluka, Sigma)
- Phosphate buffered saline (Fluka, Sigma)
- Salts for ACSF preparation (Fluka, Sigma)

- Sucrose (Fluka, Sigma)
- Methanol puriss. (Fluka, Sigma)
- Paraformaldehyde (EM grade, Nr. 19208; Electron Microscopy Sciences)
  ! CAUTION Harmful if inhalated. To be used only in a ventilated hood
- Embedding medium for frozen sectioning (e.g., M1 embedding matrix, #1310; Thermo Shandon, Pittsburgh, PA 15275)
- Parafilm (Pechiney Plastic Packaging, Chicago, IL 60631)
- Cyanoacrilate glue
- Coated microscope slides (gelatin-coated or "Superfrost" slides)
- Coverslips for microscopy (Karl Hecht Assistent Gmbh)
- Mounting medium for fluorescent sections (Mowiol, Dako Fluorescent Mounting Medium)
- Primary antibodies against cell markers (see Table 1)
- Primary antibodies against presynaptic proteins (see Table 1)
- Primary antibodies against $GABA_A$ receptor subunits (see Table 1)
- Secondary antibodies coupled to distinct fluorochromes emitting around 500, 600, and 700 nm (Jackson Immunoresearch, Molecular Probes)
- Normal serum of the host species of the secondary antibodies (Serotec)
- Triton X-100 (Fluka)

EQUIPMENT

- Laser scanning confocal microscope
- Cryostat
- Gas tank (95% O_2/5% CO_2) (option C)
- Vibratome (option C)
- Water repellent slide marker (Pap-Pen or equivalent)

REAGENT SETUP

Fixative

Dissolve paraformaldehyde in warm dH_2O (up to 60ºC); add 2 drops NaOH 1 N /100 ml; make final dilution in 0.1 M sodium phosphate buffer (0.1%, 2%, or 4%), allow cooling at

room temperature, adjust the pH to 7.4 and filter. ▲CRITICAL Make up fresh on day of experiment; the solution can be aliquoted and frozen for future use.

Artificial cerebrospinal fluid (ACSF) 10X

This stock solution is made by diluting 125 mM (73.05 g/l) NaCl, 26 mM (21.84 g/l) $NaHCO_3$, 1.25 mM (1.73 g/l NaH_2PO4 monohydrate), 2.5 mM (3.72 g/l) KCl and 1 mM $MgCl_2$ (corresponding to 2.04 ml from a 4.9 M stock solution) in dH_2O. Store at 4°C up to several weeks.

ACSF 1X

To make one liter, dilute 100 ml of 10X ACSF in 900 ml dH_2O and add 2 g glucose (11 mM final concentration) and 6.6 ml $CaCl_2$ (1 Osm Stock; final concentration 2.5 mM). The osmolarity should be around 320 mOsm, pH 7.4; adjust with glucose or dH_2O as necessary. ▲CRITICAL Make up fresh on day of experiment.

Cryoprotectant

Dissolve 30% sucrose (w/v) in 0.1 M sodium phosphate buffer, pH 7.4, and refrigerate to 4ºC. ▲CRITICAL Make up fresh on day of experiment.

Blocking solution

Dilute 10% normal serum of the host species of the secondary antibodies (typically goat or donkey) and 0.2 % Triton X-100 in phosphate buffered saline (PBS). ▲CRITICAL Make up fresh on day of experiment.

Primary antibodies

This protocol requires primary antibodies raised in different species (e.g. mouse, rabbit, guinea pig) and secondary antibodies devoid of cross-reactivity and all raised in the same species. They are diluted at the appropriate concentration (to be determined by the end-user) in phosphate buffered saline (PBS) that contains 10% normal serum of the species in which the secondary antibodies were raised and 0.2% Triton X-100. ▲CRITICAL Make up fresh on day of experiment.

Secondary antibodies

Secondary antibodies coupled to a large variety of fluorochromes are available, which provide satisfactory quantum yield and limited bleaching. Carbocyanine dyes (Cy2, Cy3, Cy5) and Alexa fluorochromes (e.g. Alexa-Fluor 488, 543, etc) are largely comparable and suitable for these applications. For confocal laser scanning microscopy, typical fluorochromes have maximal excitation spectrum around 480, 540, and 630 nm. These emission spectra can be separated without any overlap using sequential detection of each fluorochrome with appropriate band pass filters.

Dilute secondary antibodies at the appropriate concentration (to be determined by the end-user) in PBS that contains 10% normal serum of the host species. ▲CRITICAL Make up fresh on day of experiment.

Procedure

Tissue preparation

1. There are two basic preparation methods (Fig. 1). Use option A for preparing sections from fresh frozen brain, and options B and C for preparing sections from dissected tissue slabs and from living tissue slices, respectively.

(A) Sections from fresh frozen brain

(i) Anesthetize a mouse with a volatile anesthetic and decapitate it with a sharp scissor.
! CAUTION Cervical dislocation often damages caudal brain structures.

(ii) Rapidly excise the brain and place it on a cold surface (Petri dish placed on ice).

(iii) Dissect the region of interest with a scalpel, mount it on a piece of cork (2 x 2 x 0.2 cm) using a drop of PBS, and freeze with powdered dry ice.

(iv) After 3 min wrap the frozen block air-tight in aluminum foil and store it at −80ºC.
! CAUTION The tissue should be used within a few weeks, due to dehydration in the freezer.

(v) Set the temperature of the cryostat chamber to -20ºC and that of the knife to -22ºC.

(vi) Place the frozen tissue in the chamber of the cryostat and allow the temperature to equilibrate (for at least 30 min).

(vii) Mount the cork on the specimen holder and orient for cutting.

(viii) Cut 12-16 μm-thick cryosections, mount them onto gelatin-coated slides (kept at room temperature) and allow to air dry for exactly 30 sec at room temperature. Use a slide marker to draw a hydrophobic ring around the sections and store at –20°C in the cryostat until cutting is completed.

▲CRITICAL STEP Best results are obtained when the tissue can be further processed immediately after cutting; if necessary, sections can be stored up to a few days at –20°C. **?** TROUBLESHOOTING: see Table 2.

(ix) Fix samples using methods described in Box 1.

(x) After fixation and rising are complete, continue immediately with the immunofluorescence staining starting from Step 2.

BOX 1. ***Fixation methods***

For fixation, there are three possible methods (option A1, A2, or A3). Option A1 provides the weakest fixation and should be used for antigens highly sensitive to fixation; Option A2 is well suited for most purposes; option A3 is optimal for tissue from young animals.

A1 Microwave-assisted fixation with paraformaldehyde

1. Place 3-4 slices in a Petri dish of 14cm diameter and add 50ml fixative (containing 0.1% paraformaldehyde)
2. Irradiate for 30 sec in a microwave oven (600 – 900 W)
3. Rinse and store the slides with PBS

A2 Fixation with paraformaldehyde

1. Dip the slides in fixative containing 2% paraformaldehyde (4 ºC) for 90 sec
2. Rinse the slides with PBS and store in PBS

A3 Brief fixation with methanol

1. Dip the slides in cold methanol (-20ºC) for 30 sec
2. Rinse the slides with PBS and store in PBS

(B) Dissected tissue slabs

● TIMING This procedure requires 2 consecutive days (Fig. 1). On day 1, preparation, fixation and cryoprotection of tissue slabs occurs; on day 2, sectioning occurs.

(i) Anesthetize a mouse with a volatile anesthetic and decapitate it with a sharp scissor.

! CAUTION Cervical dislocation often damages caudal brain structures.

(ii) Rapidly excise the brain and place it on a cold surface (a Petri dish placed on ice)

(iii) Dissect the regions of interest, cut tissue slabs of 1-2 mm thickness with a sharp scalpel and rinse briefly in ice-cold PBS (alternatively, a tissue chopper can be used)

▲CRITICAL STEP This procedure should be completed as fast as possible.

(iv) Dip the tissue slabs in ice-cold fixative (2-4% paraformaldehyde; 5-10 ml per slab) and fix for 10-45 min; the fixation time depends on the type of tissue and primary antibodies used.

(v) Rinse extensively (3 times 5 min, with agitation) with ice-cold PBS.

(vi) Transfer to cryoprotectant (10 ml per slab) and store overnight at 4ºC.

■ PAUSE POINT The tissue should not be stored in cryoprotectant longer than overnight.

(vii) Section samples according to the method described in Box 2.

■ PAUSE POINT Sections from option B can be stored for up to 2 weeks at -20 °C.

(viii) Proceed to Step 2.

(C) Living tissue slices

● TIMING This procedure requires 2 consecutive days (Fig. 1). On day 1, preparation of living tissue slices, fixation and cryoprotection occurs; on day 2, sectioning occurs.

(i) Pour about 100 ml ACSF in a glass beaker placed on ice and about 100 ml in another beaker placed in a water bath pre-warmed to 33°C; oxygenate both with 95% O_2/5% CO_2.

(ii) Anesthetize a mouse with a volatile anesthetic and decapitate it with a sharp scissor.

! CAUTION Cervical dislocation often damages caudal brain structures.

(iii) Rapidly excise the brain and place it in ice-cold ACSF oxygenated with 95% O_2/5% CO_2 for 2 min.

▲CRITICAL STEP Speed is crucial for optimal results; all instruments should be ice-cold.

(iv) Block the brain in desired orientation and mount on the vibratome stage with cyanoacrylate glue; keep submerged with ice-cold ACSF oxygenated with 95% O_2/5% CO_2 for slicing.

(v) Cut 300 μm-thick slices in the desired plane with the vibratome (at a high frequency and low speed) and let them recover for about 20 min in oxygenated ACSF at 33°C.

▲CRITICAL STEP Living tissue should be used immediately after sectioning.

(vi) Transfer the slices into fixative (3 ml per slice) and fix for 10-20 min (the duration depends on primary antibodies and region of interest).

(vii) Rinse extensively (3 times for 20 min, with low agitation) in PBS.

(viii) Transfer to cryoprotectant (3 ml per slice) and store overnight at 4°C

■ PAUSE POINT The tissue should not be stored in cryoprotectant longer than overnight.

BOX 2. ***Tissue slice sectioning***

1. Set the temperature of the cryostat chamber to –20°C and the knife temperature to –22°C
2. Place a large drop of embedding medium onto a metal specimen holder
3. Once the embedding medium is frozen, cut the surface to make it flat
4. Wrap a glass slide with parafilm and place the slab on this surface after rinsing it shortly in ice-cold PBS
5. Immediately transfer the slab onto the frozen embedding medium flat surface and let it freeze
6. Cover with aluminum foil and allow temperature equilibration for at least 30 min
7. Cut 12-16 μm-thick cryosections, mount them onto gelatin-coated slides (kept at room temperature) and allow to air dry for 30-60 sec at room temperature. Use a Pap-pen to draw a hydrophobic ring around the sections and store at –20°C in the cryostat until cutting is completed.

▲CRITICAL STEP Even better results can be obtained with delicate tissue (e.g., retina, young animals) by mounting the sections on cold slides (kept inside the cryostat). In this case, the tissue should be equilibrated with cryoprotectant and mounting medium (1:1; 30 min) before freezing and should be covered with mounting medium before cutting.

? TROUBLESHOOTING: see Table 2.

(ix) Section samples according to the method described in Box 2.

▲CRITICAL STEP Sections from option C are best processed immediately for immunofluorescence staining.

(x) Proceed to step 2.

Immunofluorescence staining: Incubation in primary antibodies

2. Pipet 100-300 µl blocking solution on each slide and incubate for 60 min.

! CAUTION The sections should never dry during the entire procedure.

3. In the mean time, prepare primary antibody solutions (see Reagents setup)

4. Discard the blocking solution and carefully dry the glass slides outside of the repellent ring.

5. Pipet 100-300 µl primary antibody solution inside of the water repellent ring, making sure to cover all sections on the glass slide

6. Place all slides into a humid chamber and incubate overnight at 4ºC with gentle agitation; shorter incubation at room temperature (e.g., 3 h) is also possible but the penetration of antibodies into the sections might be reduced.

■ PAUSE POINT The incubation in primary antibodies should not last longer than overnight.

Immunofluorescence staining: Incubation in secondary antibodies

7. Rinse the slides with PBS (3 x 10 min with gentle agitation).

8. In the mean time, prepare secondary antibody solutions (see Reagents setup).

9. Carefully dry the glass slides outside of the repellent ring.

10. Pipet 200 - 400 µl secondary antibody solution over the sections.

11. Incubate for 60 min at room temperature.

12. Rinse the slides with PBS (at least 3 x 10 min with agitation).

13. Discard excess buffer (without drying the sections) and cover with appropriate mounting medium using coverslips calibrated for microscopy.

■ PAUSE POINT The staining procedure is now complete. Store the slides at 4ºC, in the dark, for up to 2 weeks. Allow for the mounting medium to polymerize (overnight at 4ºC) before examination at the microscope.

TABLE 1. ***Comparing subcellular distribution patterns of selected presynaptic and postsynaptic proteins***

Target protein	Antibody	Perfusion-fixed	Option A: Fresh-frozen brain	Option B: Dissected tissue slabs	Option C: Living tissue slices
$GABA_A$ receptor subunits	Custom-made guinea pig sera against α1, α2, α3, α5, and γ2 subunits[31]	Uniform cell-surface staining	Clustered, postsynaptic staining [19]	Clustered, postsynaptic staining [32, 36]	Clustered, postsynaptic staining
$GABA_B$ receptor subunits	Rabbit anti-GABABR1 subunit (Chemicon International)	Uniform cell-surface staining	Uniform cell surface staining	Uniform cell-surface staining	Not tested
NMDA receptor subunits	Custom-made guinea pig antibodies NR2A and NR2B[19]; rabbit anti-NMDAR1 subunit (Chemicon Internacional)	Intracellular staining, most prominent in soma	Clustered, postsynaptic staining [19]	Not tested	Not tested
AMPA receptor subunits	Rabbit anti-GluR1 and GluR2,3 antibodies (Chemicon Internacional)	Mainly intracellular staining	Mainly intracellular staining (unpublished)	Mainly intracellular staining (unpublished)	Not tested
Gephyrin	Mouse monoclonal antibody (Synaptic Systems)	Partially clustered at postsynaptic sites; high background in forebrain	Clustered, postsynaptic staining [36]	Clustered, postsynaptic staining	Clustered, postsynaptic staining
PSD-95	Mouse monoclonal antibody (Chemicon, Internacional)	Diffuse intracellular staining	Not tested	Clustered, postsynaptic staining	Not tested
Bassoon	Mouse monoclonal antibody (AMS Biotechnology)	Punctate staining of synaptic terminals	Non-specific staining	Partial staining of synaptic terminals	Not tested

Synapsin 1	Rabbit anti-synapsin 1 (Molecular Probes)	Punctate staining of synaptic terminals	Non-specific staining	Partial staining of synaptic terminals	Not tested
Vesicular GABA transporter	Rabbit anti-vGAT (Synaptic Systems)	Punctate staining of synaptic terminals	Partial staining of synaptic terminals	Partial staining of synaptic terminals	Punctate staining of synaptic terminals
Parvalbumin	Mouse monoclonal antibody (SWant); Rabbit anti-parvalbumin (Immunostar)	Prominent intracellular staining of somata, dendrites and axons	Detectable only in isolated neurons	Labeling of somata and dendrites	Prominent intracellular staining of somata, dendrites and axons
Calbindin	Mouse monoclonal antibody (SWant) Rabbit anti-calbindin (Immunostar)	Prominent intracellular staining of somata and dendrites	Not detectable	Labeling of somata and dendrites	Prominent intracellular staining of somata, dendrites and axons
Tyrosine hydroxylase	Rabbit anti-tyrosine hydroxylase (Chemicon International)	Staining of somata, dendrites and axons	Not detectable	Labeling of somata and dendrites	Prominent intracellular staining of somata, dendrites and axons
Serotonin	Rabbit anti-serotonin (Immunostar)	Staining of somata, dendrites and axons	Not detectable	Not tested	Prominent intracellular staining of somata, dendrites and axons
eGFP	GAD67-eGFP transgene and GlyT2-eGFP transgene	Staining of somata, dendrites and axons	Not detectable	Weak staining of somata and dendrites; moderate background	Prominent intracellular staining of somata, dendrites and axons

Microscopy and image analysis

14. Evaluation of immunofluorescence staining patterns can be done by epifluorescence microscopy. Imaging of synaptic structures is best achieved by confocal laser scanning microscopy. There is no instrument-specific requirement for analyzing tissue sections prepared according to these three protocols. For dual and triple labeling experiment, the

microscope has to be equipped with the corresponding lasers and fluorescence filter sets. A high resolution objective (numerical aperture >1.3) is necessary for optimal resolution. Acquisition of multiple labeling has to be done sequentially with appropriate band pass filters to avoid cross talk between fluorochromes. Figures 2 and 3 were prepared using a confocal microscope LSM 510 Meta (Zeiss AG) and the image analysis software Imaris (Bitplane). Background was subtracted and a low pass filter ('edge preserving' filter) was applied. All images are shown by superposition of the different color channels using the maximal intensity projection mode (stack of 5-30 confocal images spaced by 0.25 – 0.5 μm).

? TROUBLESHOOTING: see Table 2.

Timing

Option A

Tissue preparation (1A, i –iv), 10 min per mouse

Sectioning (1A, v-viii), 60-90 min per brain

Fixation (1A ix; Box 1, A1), 30 min for 20 slides

Fixation (1A ix; Box 1, A2), 20 min for 20 slides

Fixation (1A, ix; Box 1, A3), 20 min for 20 slides

Option B

Tissue preparation (1B, i-iii), 10 min per mouse

Fixation and cryoprotection (1B, iv-vi), 30-60 min depending on the duration of fixation

Sectioning (Box 2) 60-90 min per tissue slice

Option C

Tissue preparation (1C, i-v), 60-90 min per mouse

Fixation and cryoprotection (1C, vi-viii), 90 - 120 min

Sectioning (Box 2) 60-90 min per tissue slice

Incubation in primary antibodies (i-v), 90 min and overnight incubation

Incubation in secondary antibodies (vi-xii), 150 min

TABLE 2. ***Troubleshooting table***

Step	Problem	Possible solution
A (viii) or Box 2	The sections are wrinkled or rolled during cutting	(i) Perfectly align the glass plate with the knife (ii) Change the thickness of the section
14	The sections do not adhere to the slides	(i) The slide is too cold (ii) Embed tissue with mounting medium prior to cutting (iii) Store the sections overnight at -20ºC prior to staining (iv) Make sure that the gelatin coating of the slides is uniform (v) If possible, prepare larger tissue slabs or slices surrounding the region of interest (large sections adhere better)
14	The level of background fluorescence is too high	(i) The quality of frozen sections is suboptimal (ii) Make sure that the tissue remains humid during the entire staining procedure (iii) The tissue might have been stored for too long in the freezer (iv) The fixative was not appropriate
14	The distribution of presynaptic or postsynaptic markers is not clustered	(i) Shorten the duration of brain dissection (ii) Fixative has to be freshly prepared (iii) Avoid storing sections for longer than a few weeks at -20ºC (iv) Adjust the duration of fixation to the primary antibodies used (v) Reduce the concentration of primary antibodies (vi) Increase the number and duration of washing steps during the immunostaining procedure

? TROUBLESHOOTING

General comments

These procedures require a good practice in preparing cryostat sections and in performing and evaluating immunofluorescence experiments. The specificity of primary antibodies has to be well established; conventional immunohistochemical staining of perfusion-fixed tissue can be used for this purpose; option A is also well suited for a general screening. The use of mouse monoclonal antibodies on mice tissue sections may result in background from the secondary antibodies (e.g. non-specific labeling of cell nuclei or blood vessels). Cross-reactivity between secondary antibodies can be limited by using affinity-purified secondary antibodies when necessary. In mice, lipofucsin pigments, which are strongly

fluorescent over a broad spectrum of excitation, are formed in neurons at a younger age than in rats and are potentially confounded with "clustered" immunolabeling; if possible, use animals younger than 12 weeks. Further troubleshooting advice can be found in Table 2.

Anticipated results

These protocols were developed for immunofluorescence in CNS tissue with four specific goals: (i) overcome the limited detection of postsynaptic proteins caused by tissue fixation while maintaining a good morphological preservation; (ii) distinguish pre- and postsynaptic compartments by apposition of immunofluorescent clusters of pre- and postsynaptic molecules; (iii) investigate the distribution of identified presynaptic terminals and postsynaptic markers in neurochemically defined neurons; (iv) combining high resolution immunofluorescence imaging with biochemical or functional analyses performed on the same animal or tissue slices. The procedures were optimized for inhibitory synapses, but previous results indicated that at least options A and B are also suitable for studying other neurotransmitter systems, including glutamatergic synapses [19] (Table 1).

It should be emphasized that epitopes differ widely in their sensitivity to fixation and that all proteins mentioned here are readily detected in perfusion-fixed tissue (Table 1). However, epitope-masking in specific subcellular compartments, notably in the postsynaptic density, can occur in perfusion-fixed tissue. This effect is illustrated in Fig. 2a-b, which compares the subcellular distribution of the $GABA_A$ receptor α1 subunit in mitral cells of the olfactory bulb in perfusion-fixed tissue[31] and in tissue processed according to option B. In both cases, a prominent immunofluorescence, reflecting the high density of this protein in mitral cells, is evident. However, the clustered distribution of α1-$GABA_A$ receptors at postsynaptic sites is detectable only in weakly fixed tissue[32,33]. Such a dramatic difference is not predictable and does not occur for all postsynaptic membrane proteins. For instance, staining of receptors present at low density at synaptic sites, such as $GABA_B$ receptors, is not improved using option 1A compared to perfusion-fixed tissue (Table 1). These unpredictable effects of fixation strength on the apparent subcellular distribution of synaptic proteins make it difficult to ascertain that a given staining pattern corresponds to the native distribution of these proteins. Additional information, notably

ultrastructural evidence, should be used to evaluate staining results. For instance, the selective distribution of gephyrin in symmetric (i.e. inhibitory) synapses seen by electron microscopy [33-36] provides indirect evidence for the postsynaptic localization of proteins colocalized with gephyrin in immunofluorescence experiments.

Each of the three options yields a distinctive immunofluorescence staining of $GABA_A$ receptor subunits and gephyrin in sections from rodent brain tissue, as illustrated in Fig. 2b-g and Fig. 3a-e, g-i. Option A provides a highly sensitive detection of $GABA_A$ receptors and gephyrin and allows the investigation of multiple brain regions or animals in a single experiment, because of the relative ease and speed of tissue preparation (Fig. 1). The discrete nature of clusters makes them readily amenable for quantification in two- or three-dimensional images. This protocol has been applied extensively to study $GABA_A$ and glycine receptors [32, 36-40], NMDA receptors [19], as well as postsynaptic proteins of the dystrophin glycoprotein complex [41, 42]. Its value is limited for the detection of presynaptic markers [37], because the immunofluorescence signal is generally blurred or almost undetectable (Table 1).

Option B provides a good signal-to-noise ratio for the detection of several postsynaptic proteins (Fig. 2f), although the optimal fixation time may vary for different antibodies to be used in multiple labeling experiments. This protocol also allows detection of cell markers and presynaptic molecules (e.g., synapsin 1, vGAT), as illustrated in Fig. 2g. However, this observation cannot be generalized and needs to be tested for different brain regions and markers (Table 1). Because tissue slices are fixed by immersion, there may be variability among different cryostat sections obtained from the same slice. However, in our common practice this has never been a major impediment. If required, the use of three dimensional reconstruction from stacks of confocal images provides unequivocal evidence for spatial apposition between $GABA_A$ receptor clusters and dendrites immunopositive for a neuronal markers such as calbindin (Fig. 2f), despite the limited resolution of confocal laser scanning microscopy along the z-axis.

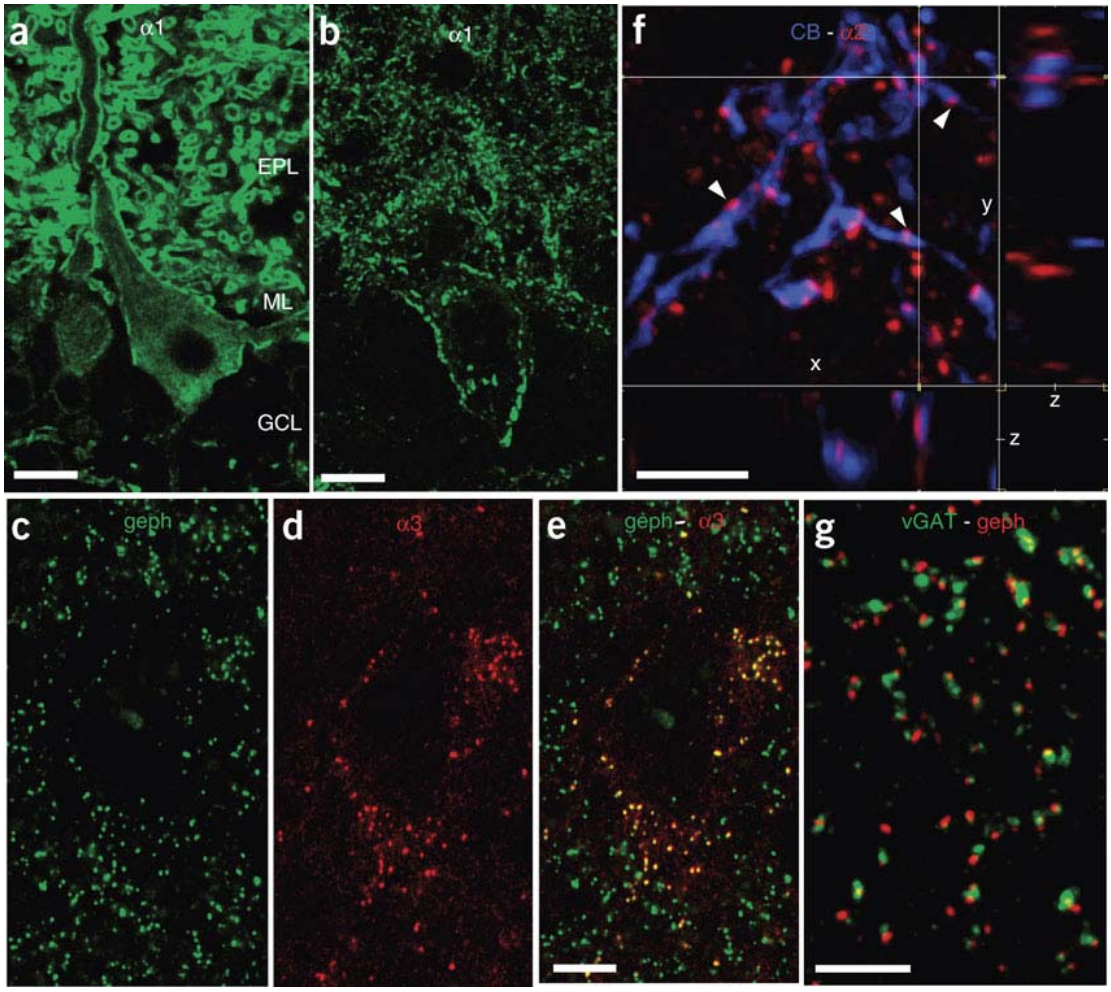
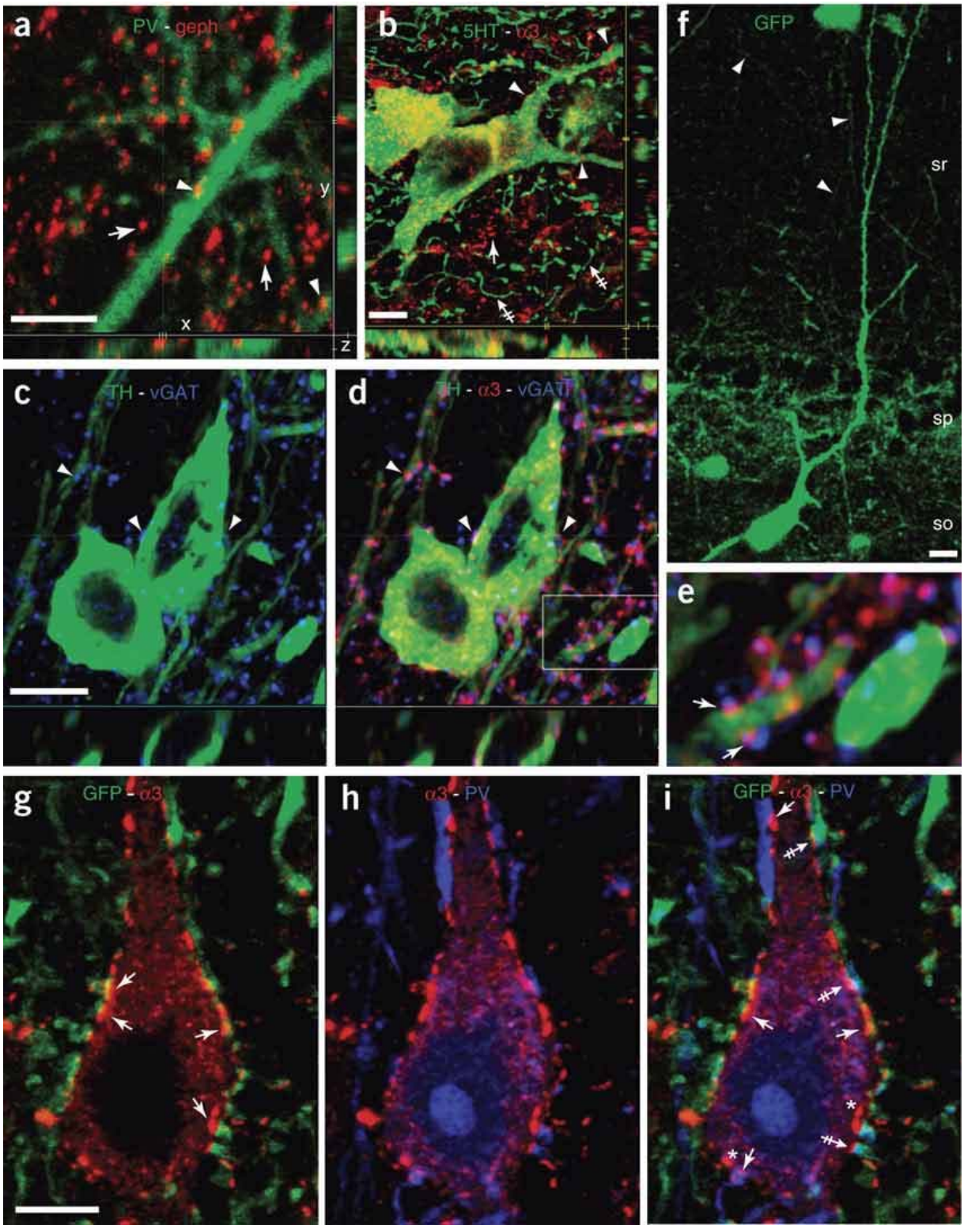


Figure 2. Examples of double immunofluorescence staining of mouse and rat brain tissue with options A and B compared to perfusion-fixed tissue. Panels a and b show a comparison of $GABA_A$ receptor α1-subunit immunofluorescence in mitral cells of the mouse olfactory bulb in perfusion-fixed tissue (a) and in tissue processed according to option B (b) (guinea pig antiserum against an N-terminal peptide[31], diluted 1:5000 and 1:40'000, respectively). The strong fixation that is produced with perfusion results in a uniform surface labeling of the soma and dendrites (many of them are transversally cut in panel a), without any evidence for postsynaptic aggregation. By contrast, staining of weakly fixed tissue results in a clustered pattern corresponding to postsynaptic aggregates. EPL, external plexiform layer; CGL, granule cell layer; ML, mitral cell layer. Panels c-e show double-labeling for gephyrin (geph; green, diluted 1:700) and the $GABA_A$ receptor α3-subunit (red; guinea pig antiserum against an N-terminal peptide[31], diluted 1:3'000) in the CA1 region of the mouse hippocampus (stratum oriens), using option A. Panel c illustrates the clustered distribution of gephyrin, which is selectively localized in the postsynaptic density of GABAergic synapses. The α3 subunit is present in a subset of interneurons and is most concentrated in clusters on their soma and proximal dendrites (d). Superposition of both panels (e) demonstrates the postsynaptic localization of α3-$GABA_A$ receptor clusters by their precise colocalization with gephyrin. Panel f shows the distribution of $GABA_A$ receptor α2-subunit clusters (red; affinity-purified guinea pig antiserum against an N-terminal peptide[31], diluted 1:1000) on the dendrites of a calbindin- (CB)-positive periglomerular cell (blue; rabbit antibody, diluted 1:3'000) in the rat olfactory bulb (option B). The stack of confocal sections was projected in three orthogonal planes at the level of the lines. Note the reduced resolution along the z-axis, resulting in an ovoid cluster shape. Most α2-$GABA_A$ receptor clusters in this image are located on the labeled dendrites (arrowheads). Panel g shows the close apposition of vGAT-positive GABAergic terminals (green) with gephyrin clusters (red) in the molecular layer of the mouse cerebellum (option B); note that the two markers are not colocalized but closely apposed, reflecting the spatial separation of presynaptic and postsynaptic elements. Scale bars for all panels, 10 μm.

Option C provides an excellent preservation of soluble proteins, such as eGFP, neurotransmitters, and presynaptic markers, while retaining the strong and highly sensitive staining of postsynaptic proteins (Fig. 3; table 1). So, the distribution of synapses can be analyzed and quantified on identified neurons, as shown for interneurons expressing parvalbumin (Fig. 3a,h-i), serotonin (Fig. 3b), tyrosine hydroxylase (a marker of catecholaminergic neurons; Fig. 3c-e), GAD67-eGFP (a marker of GABAergic neurons; Fig. 3f), and GlyT2-eGFP (glycine transporter type 2, a marker of glycinergic neurons; Fig. 3g, i). The preservation of small neurotransmitter molecules, such as serotonin, is excellent and comparable to that seen in perfusion-fixed tissue. In contrast, there is a reduction in the intensity of eGFP compared to perfusion-fixed tissue, but, as illustrated in Fig. 3f-i, the remaining signal is sufficient for unambiguous detection of axons and synaptic terminals.

Figure 3. Examples of double and triple immunofluorescence staining of mouse brain tissue with option C. Panel a shows clusters of gephyrin (red; diluted 1:700) in relation to parvalbumin- (PV)-positive dendrites (green; mouse monoclonal, diluted 1:10'000) in the CA1 stratum radiatum. A projection in three orthogonal planes at the level of the thin lines is shown. Arrowheads point to gephyrin clusters on dendrites, representing presumptive GABAergic postsynaptic sites. Many gephyrin clusters are not associated with PV-positive profiles (arrows). Panel b shows clusters of $GABA_A$ receptor α3-subunits (red; guinea pig antiserum against an N-terminal peptide[31], diluted 1:3'000) in relation to a serotonin- (5HT)-positive neuron (green; rabbit antibody, diluted 1:5'000) in the nucleus raphe magnus; only few, small clusters are located on this cell (arrowhead), whereas larger clusters are found in the surrounding neuropil (arrows). Note that 5HT-positive axons (crossed arrows) are not associated with α3-$GABA_A$ receptors, as expected. The yellow color of the cell body results from intracellular staining of the α3 subunit. Panels c-e show GABAergic terminals, stained for vGAT (blue; rabbit affinity-purified antibody, Synaptic Systems, diluted 1:3'000) apposed to α3-$GABA_A$ receptor clusters (red) on tyrosine hydroxylase (TH)-positive cells (green; rabbit antibody, diluted 1:10'000) in the substantia nigra (arrowheads). The boxed area is enlarged in panel e to highlight the spatial separation between pre- and postsynaptic markers (arrows). Note the higher density of synapses on dendrites compared to the soma of dopaminergic cells. Expression of the α3 subunit by these dopaminergic neurons is also evident in the soma, which appears yellow in the overlay (panel d). Panel f shows the eGFP labeling of GABAergic interneurons and axons in the CA1 region of the hippocampus (under control of the GAD67 promoter); note that eGFP is well preserved in fine structures (arrowheads), even without amplification by immunofluorescence. Panels g-i show heterogeneity of presynaptic input onto α3-$GABA_A$ receptors (red) in a neuron of the pontine reticular formation visualized by triple staining with GlyT2-eGFP (green) and PV. Panel g illustrates GlyT2-eGFP positive terminals, representing glycinergic synapses, terminating onto α3 subunit clusters (arrows); panel h shows that the postsynaptic cell expresses PV and receives a prominent innervation from PV-positive terminals; panel i illustrates that individual α3 subunit clusters are either alone (star) or associated with single (arrows) or double-labeled (crossed arrows) terminals. Scale bars for all panels represent 10 μm.


a
PV - geph
y
x
z
b
5HT - α3
f
GFP
sr
sp
so
c
TH - vGAT
d
TH - α3 - vGAT
e
g
GFP - α3
h
α3 - PV
i
GFP - α3 - PV

The difference between a neurotransmitter and eGFP probably stems from the fact that the latter is present as a soluble protein in the cytoplasm, without any compartmentalization, for example in synaptic vesicles. eGFP-fusion proteins, or membrane-anchored eGFP-reporter genes, would not be affected by rapid outward diffusion in weakly fixed tissue.

The high signal-to-noise ratio obtained in tissue sections processed with option C allows a clear and unambiguous spatial separation of pre- and postsynaptic markers (Fig. 3e, i), as well as a distinction between different populations of presynaptic terminals innervating a particular cell compartment. In the example shown in Fig. 3g-i, at least four types of GABAergic synapses containing the α3 subunit can be distinguished based on markers of presynaptic terminals (GlyT2-eGFP, representing glycinergic terminals; parvalbumin; both markers together; no marker). The major limitations of option C are that only a few animals at a time can be processed and the results can be quite variable because they depend critically on the speed of the brain dissection and the quality of the cryostat sections.

The use of confocal laser scanning microscopy provides spatial segmentation of images and three-dimensional reconstruction, facilitating the quantification of structures in space. Quantifying the number of presumptive presynaptic or postsynaptic sites of individual neurons identified by the expression of a selective marker is therefore feasible. Furthermore, slices prepared according to option C can be utilized first for electrophysiological analysis; using an intracellular dye to label the recorded neuron(s), correlative microscopy is then readily available. Likewise, correlative light and electron microscopy is possible using alternate slices for immunofluorescence and immunogold electron microscopy. Option C can therefore be expected to help overcome barriers among disciplines and provide detailed morphological parameters of functionally characterized neurons.

Acknowledgements

The authors wish to thank C. Sidler for excellent technical assistance and L. Viltono for providing Figure 2g. The work was supported by the Swiss National Science Foundation, grant Nr. 3100A0-108260 to J.-M.F., and the Italian MIUR grant (PRIN 2005059123 002) to M.S.-P.

References

1. Racz B., Blanpied T.A., Ehlers M.D., Weinberg R.J. Lateral organization of endocytic machinery in dendritic spines. Nature Neurosci. 7, 917-918 (2004).
2. Triller A., Cluzeaud F., Pfeiffer F., Betz H., Korn H. Distribution of glycine receptors at central synapses: an immunoelectron microscopy study. J. Cell Biol. 101, 683-688 (1985).
3. Somogyi P. Synaptic organization of GABAergic neurons and $GABA_A$ receptors in the lateral geniculate nucleus and visual cortex. In Neural mechanisms of visual perception. ed. (Lam DKT, Gilbert CD, editors) 35-62 (Houston: Portfolio Publishing Co; 1989).
4. Lujan R., Nusser Z., Roberts J.D., Shigemoto R., Somogyi P. Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. Eur. J. Neurosci. 8, 1488-1500 (1996).
5. Landis D.M. Membrane and cytoplasmic structure at synaptic junctions in the mammalian central nervous system. J. Electr. Microsc. Tech. 10, 129-151 (1988).
6. Harlow M.L., Ress D., Stoschek A., Marshall R.M., McMahan U.J. The architecture of active zone material at the frog's neuromuscular junction. Nature 409, 479-84 (2001).
7. Dick O., et al. The presynaptic active zone protein bassoon is essential for photoreceptor ribbon synapse formation in the retina. Neuron 37, 775-786 (2003).
8. Baron M.K., et al. An architectural framework that may lie at the core of the postsynaptic density. Science 311, 531-535 (2006).
9. Ottersen O.P., Landsend A.S. Organization of glutamate receptors at the synapse. Eur. J. Neurosci. 9, 2219-2224 (1997).
10. Cowan W.M., Sudhof T.C., Stevens C.F. Synapses. Baltimore: Johns Hopkins University Press; 2001.
11. Phillips G.W., Bridgman P.C. Immunoelectron microscopy of acetylcholine receptors and 43 KD protein after rapid freezing, freeze-substitution, and low-temperature embedding in Lowicryl K11M. J. Histochem. Cytochem. 39, 625-634 (1991).

12. Nusser Z., et al. Immunocytochemical localization of the α1 and β2/3 subunits of the $GABA_A$ receptor in relation to specific GABAergic synapses in the dentate gyrus. Eur. J. Neurosci. 7, 630-646 (1995).
13. Kulik A., et al. Compartment-dependent colocalization of Kir3.2-containing K^+ channels and $GABA_B$ receptors in hippocampal pyramidal cells. J. Neurosci. 26, 4289-4297 (2006).
14. Hagiwara A., Fukazawa Y., Deguchi-Tawarada M., Ohtsuka T., Shigemoto R. Differential distribution of release-related proteins in the hippocampal CA3 area as revealed by freeze-fracture replica labeling. J. Comp. Neurol. 489, 195-216 (2005).
15. Rash J.E., Yasumura T., Dudek F.E. Ultrastructure, histological distribution, and freeze-fracture immunocytochemistry of gap junctions in rat brain and spinal cord. Cell Biol. Int. 22, 731-749 (1998).
16. Rash J.E., et al. Ultrastructural localization of connexins (Cx36, Cx43, Cx45), glutamate receptors and aquaporin-4 in rodent olfactory mucosa, olfactory nerve and olfactory bulb. J. Neurocytol. 34, 307-341 (2005).
17. Wouterlood F.G., Bockers T.M., Witter M.P. Synaptic contacts between identified neurons visualized in the confocal laser scanning microscope. Neuroanatomical tracing combined with immunofluorescence detection of post-synaptic density proteins and target neuron-markers. J. Neurosci. Meth. 128, 129-142 (2003).
18. Koulen P., Sassoè-Pognetto M., Grünert U., Wässle H. Selective clustering of $GABA_A$ and glycine receptors in the mammalian retina. J. Neurosci. 16, 2127-2140 (1996).
19. Fritschy J.M., Weinmann O., Wenzel A., Benke D. Synapse-specific localization of NMDA- and $GABA_A$-receptor subunits revealed by antigen-retrieval immunohistochemistry. J. Comp. Neurol. 390, 194-210 (1998).
20. Geiman E.J., Zheng W., Fritschy J.M., Alvarez F.J. Glycine and $GABA_A$ receptor subunits on Renshaw cells: relationship with presynaptic neurotransmitters and postsynaptic gephyrin clusters. J. Comp. Neurol. 444, 275-289 (2002).
21. Sassoe-Pognetto M., Wassle H., Grunert U. Glycinergic synapses in the rod pathway of the rat retina: cone bipolar cells express the α1 subunit of the glycine receptor. 14, 5131-5146 (1994).

22. Melone M., Burette A., Weinberg R.J. Light microscopic identification and immunocytochemical characterization of glutamatergic synapses in brain sections. J. Comp. Neurol. 492, 495-509 (2005).

23. Watanabe M., et al. Selective scarcity of NMDA receptor channel subunits in the stratum lucidum (mossy fibre-recipient layer) of the mouse hippocampal CA3 subfield. Eur. J. Neurosci. 10, 478-487 (1998).

24. Nagy G.G., Watanabe M., Fukaya M., Todd A.J. Synaptic distribution of the NR1, NR2A and NR2B subunits of the N-methyl-d-aspartate receptor in the rat lumbar spinal cord revealed with an antigen-unmasking technique. Eur. J. Neurosci. 20, 3301-3312 (2004).

25. Barnard E.A., et al. International Union of Pharmacology. XV. Subtypes of γ-aminobutyric $acid_A$ receptors: classification on the basis of subunit structure and function. Pharmacol. Rev. 50, 291-313 (1998).

26. Sieghart W., Ernst M. Heterogeneity of $GABA_A$ receptors: revived interest in the development of subtype-selective drugs. Curr. Med. Chem. 5, 217-242 (2005).

27. Colquhoun D., Sivilotti L.G. Function and structure in glycine receptors and some of their relatives. Trends Neurosci. 27, 337-344 (2004).

28. Lynch J.W. Molecular structure and function of the glycine receptor chloride channel. Physiol. Rev. 84, 1051-1095 (2004).

29. Kneussel M., Betz H. Receptors, gephyrin and gephyrin-associated proteins: novel insights into the assembly of inhibitory postsynaptic membrane specializations. J. Physiol. 525, 1-9 (2000).

30. Sassoè-Pognetto M., Fritschy J.M. Gephyrin, a major postsynaptic protein of GABAergic synapses. Eur. J. Neurosci. 7, 2205-2210 (2000).

31. Fritschy J.M., Mohler H. $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. J. Comp. Neurol. 359, 154-194 (1995).

32. Panzanelli P., Perazzini A.Z., Fritschy J.M., Sassoè-Pognetto M. Heterogeneity of γ-aminobutyric acid type A receptors in mitral and tufted cells of the rat main olfactory bulb. J. Comp. Neurol. 484, 121-131 (2005).

33. Triller A., Cluzeaud F., Korn H. γ-Aminobutyric acid-containing terminals can be apposed to glycine receptors at central synapses. J. Cell Biol. 104, 947-956 (1987).

34. Sassoè-Pognetto M., et al. Colocalization of gephyrin and $GABA_A$-receptor subunits in the rat retina. J. Comp. Neurol. 357, 1-14 (1995).

35. Giustetto M., Kirsch J., Fritschy J.M., Cantino D., Sassoè-Pognetto M. Localisation of the clustering protein gephyrin at GABAergic synapses in the main olfactory bulb of the rat. J. Comp. Neurol. 395, 231-244 (1998).

36. Sassoè-Pognetto M., Panzanelli P., Sieghart W., Fritschy J.M. Co-localization of multiple $GABA_A$ receptor subtypes with gephyrin at postsynaptic sites. J. Comp. Neurol. 420, 481-498 (2000).

37. Schweizer C., et al. The γ2 subunit of $GABA_A$ receptors is required for maintenance of receptors at mature synapses. Mol. Cell. Neurosci. 24, 442-450 (2003).

38. Koksma J.J., Fritschy J.M., Mack V., Van Kesteren R.E., Brussaard A.B. Differential $GABA_A$ receptor clustering determines GABA synapse plasticity in rat oxytocin neurons around parturition and the onset of lactation. Mol. Cell. Neurosci. 28, 128-140 (2005).

39. Lorenzo L.E., Barbe A., Portalier P., Fritschy J.M., Bras H. Differential expression of $GABA_A$ and glycine receptors in ALS-resistant vs. ALS-vulnerable motoneurons: possible implications for selective vulnerability of motoneurons. Eur. J. Neurosci. 23, 3161-3170 (2006).

40. Fagiolini M., et al. Specific $GABA_A$ circuits for visual cortical plasticity. Science 303, 1681-1683 (2004).

41. Knuesel I., Zuellig R.A., Schaub M.C., Fritschy J.M. Alterations in dystrophin and utrophin expression parallel the reorganization of GABAergic synapses in a mouse model of temporal lobe epilepsy. Eur. J. Neurosci. 13, 1113-1124 (2001).

42. Knuesel I., et al. Altered synaptic clustering of $GABA_A$-receptors in mice lacking dystrophin (mdx mice). Eur. J. Neurosci. 11, 4457-4462 (1999).

4. Reorganization of GABAergic circuits maintains $GABA_A$ receptor-mediated transmission onto CA1 interneurons in α1 subunit-null mice

Edith M. Schneider Gasser, Venceslas Duveau, George A. Prenosil, and Jean-Marc Fritschy

European Journal of Neuroscience (in press, 2007)

Abstract

A majority of hippocampal interneurons strongly express $GABA_A$ receptors containing the α1 subunit, suggesting that inhibitory control of interneurons is important for proper function of hippocampal circuits. Here, we investigated with immunohistochemical and electrophysiological techniques how these $GABA_A$ receptors are replaced in mice carrying a targeted deletion of the α1 subunit gene ($\alpha1^{0/0}$). Using markers of five major populations of CA1 interneurons (parvalbumin, calretinin, calbindin, neuropeptide Y and somatostatin), we show that these interneurons remain unaffected in $\alpha1^{0/0}$ mice. In triple immunofluorescence staining experiments combining these markers with the $GABA_A$ receptor α1, α2 or α3 subunit and gephyrin, we demonstrate a strong increase in α3- and α2-$GABA_A$ receptors clustered at postsynaptic sites along with gephyrin in most CA1 interneurons in $\alpha1^{0/0}$ mice. The changes were cell type-specific and resulted in an increased number of GABAergic synapses on interneurons. These adjustments were mirrored functionally by retention of spontaneous IPSCs with prolonged decay kinetics, as shown by whole-cell patch clamp recordings of CA1 interneurons. However, a significant decrease in frequency and amplitude of miniature IPSCs was evident, suggesting reduced affinity of postsynaptic receptors and/or impaired vesicular GABA release. Finally, to assess whether these compensatory changes are sufficient to protect against a pathological challenge, we tested the susceptibility of $\alpha1^{0/0}$ mice against kainic acid-induced excitotoxicity. No genotype difference was observed in the effects of kainic acid, indicating that the absence of a major $GABA_A$ receptor subtype is functionally compensated for in hippocampal interneurons by a reorganization of inhibitory circuits.

Introduction

$GABA_A$ receptors are ligand-gated chloride ion channels contributing to fast inhibitory transmission in the CNS. They form heteropentameric channels assembled from a large family of subunits encoded by distinct genes (α1-6, β1-3, γ1-3, δ, ε, θ, π) (Barnard, 2001; Sieghart & Ernst, 2005). Around 50% $GABA_A$ receptors in the brain contain the α1 subunit, along with β2 and γ2 (Benke *et al.*, 1994), and mediate the sedative, amnesic and anticonvulsant action of diazepam (Rudolph *et al.*, 1999, 2001; McKernan *et al.*, 2000; Mohler *et al.*, 2002). α1-$GABA_A$ receptors have fast decay kinetics and their expression increases during postnatal development, rendering inhibitory synaptic transmission faster (Hutcheon *et al.*, 2000; Vicini *et al.*, 2001; Bosman *et al.*, 2002).

In the hippocampal formation, all α subunit variants except α6 are expressed (Persohn *et al.*, 1992; Wisden *et al.*, 1992), contributing to multiple $GABA_A$ receptor subtypes with specific cellular and subcellular distribution patterns (Fritschy & Mohler, 1995; Nusser *et al.*, 1996; Brünig *et al.*, 2002). α1-$GABA_A$ receptors are expressed in pyramidal cells, but are especially abundant in specific populations of interneurons, notably parvalbumin-positive cells (Gao & Fritschy, 1994). These form a major population of fast-spiking interneurons and mainly comprise axo-axonic and a subset of basket cells (Bartos *et al.*, 2002; Klausberger *et al.*, 2005; Baude *et al.*, 2006). Other interneurons, notably those expressing calbindin, are apparently devoid of α1-$GABA_A$ receptors (Gao & Fritschy, 1994), but the type of receptor they carry has not been identified. It is widely assumed that the α2, α4 and α5 subunit, which are abundant in pyramidal cells, are largely absent from hippocampal interneurons (Fritschy & Mohler, 1995; Pirker *et al.*, 2000). Finally the α3 subunit, which is expressed at low levels in the hippocampus (Prenosil *et al.*, 2006), is selectively present in a subset of interneurons in CA1-CA3 stratum oriens and the hilus (Brünig *et al.*, 2002), which innervate other hippocampal interneurons and project to the septum (Gulyas *et al.*, 2003; Fritschy and Gulyas, unpublished observations).

Deletion of the α1 subunit gene by homologous recombination results in a viable phenotype (Sur *et al.*, 2001; Vicini *et al.*, 2001), due in part to compensatory alterations in the GABAergic system (Kralic *et al.*, 2002, 2006; Ogris *et al.*, 2006). In particular, a pronounced upregulation of other $GABA_A$ receptor subtypes occurs in regions depleted of α1-$GABA_A$ receptors, without change in their regional or subcellular distribution (Kralic *et*

al., 2006). However, while synaptic inhibition is retained in neocortex, hippocampal formation, olfactory bulb and cerebellar granule cells of $\alpha1^{0/0}$ mice, the kinetics of IPSCs are slower (Vicini *et al.*, 2001; Goldstein *et al.*, 2002; Barberis *et al.*, 2005; Bosman *et al.*, 2005; Lagier *et al.*, 2007), suggesting that such compensatory changes cannot fully restore the function of α1-$GABA_A$ receptors. Furthermore, a complete loss of $GABA_A$-mediated transmission can occur in specific neurons of $\alpha1^{0/0}$ mice, as shown for cerebellar Purkinje cells, for example (Kralic *et al.*, 2005a; Fritschy *et al.*, 2006). Therefore, rather than substitution of the α1 subunit by another isoform, structural reorganization of inhibitory networks contributes to retain synaptic inhibition in the brain of $\alpha1^{0/0}$ mice.

To better understand the nature of compensatory changes occurring in interneurons and their functional consequences *in vivo*, we investigated here by immunohistochemistry and whole-cell patch clamp recordings the properties of $GABA_A$ receptors expressed by hippocampal interneurons of $\alpha1^{0/0}$ mice. We focused on major populations of CA1 interneurons identified by expression of specific markers (parvalbumin (PV), calretinin (CR), calbindin (CB), neuropeptide Y (NPY) and somatostatin (SOM)) (Matyas *et al.*, 2004). First, we investigated whether the regional distribution of these interneurons and the expression of these markers are affected in mutant mice. Subsequently, changes in $GABA_A$ receptor subunit expression were examined at the subcellular level, using gephyrin as a marker of GABAergic postsynaptic sites (Sassoè-Pognetto & Fritschy, 2000; Lagier *et al.*, 2007). These results indicated a substitution of the α1 subunit by the α2 or the α3 subunit, as well as an increase in the number of postsynaptic sites, on most identified interneurons. To determine the functional correlate of these changes, spontaneous IPSCs (sIPSCs) and miniature IPSCs (mIPSCs) were recorded from interneurons in CA1 stratum oriens, along with tonic inhibition mediated by $GABA_A$ receptors. Unexpectedly, these results pointed to a decreased frequency and amplitude of mIPSCs, suggesting that the prominent morphological rearrangements seen by immunofluorescence do not fully restore GABAergic transmission on interneurons. Finally, to assess the strength of the compensatory changes occurring in the absence of α1-$GABA_A$ receptors against a pathological challenge, we tested the susceptibility of $\alpha1^{0/0}$ mice against kainic acid-induced seizures in a mouse model of temporal lobe epilepsy (Bouilleret *et al.*, 1999).

Methods

Animals

Wildtype and homozygous $GABA_A$ receptor α1 subunit knock-out ($\alpha1^{0/0}$) mice were generated on a mixed C57BL/6J-129Sv/SvJ background (see Vicini *et al.* (2001) for characterization). Wildtype and $\alpha1^{0/0}$ mice used for experiments were obtained by intercrossing heterozygous mutants ($\alpha1^{+/0}$) or homozygous mice from first-generation heterozygous intercrosses. GAD67-GFP Δneo mice (Tamamaki *et al.*, 2003) were provided by Y. Yanagawa (Gunma University Graduate School of Medicine) and maintained on a C57Bl6/J heterozygous background. Experiments were approved by the cantonal veterinary office of Zurich and performed in accordance with international guidelines on animal use and care (European Community Council Directive 86/609/EEC). All efforts were made to minimize animal suffering and the number of experimental animals used.

Kainic acid injection

Under isoflurane general anesthesia, wildtype and $\alpha1^{0/0}$ mice (n=6 per genotype) received a unilateral stereotaxic injection of 50 nl of a 20 mM solution of kainic acid (Calbiochem; San Diego, CA) in saline (i.e., 1 nmol kainic acid) into the right CA1 area of the dorsal hippocampus [coordinates with bregma as reference: anteroposterior = -1.7, mediolateral = -1.6, dorsoventral = -1.9 mm] as described (Kralic *et al.*, 2005b). The effects of kainic acid on the cytoarchitecture of the hippocampus and survival of interneurons were determined 14 days after treatment using Nissl staining and immunohistochemistry.

Immunohistochemistry

Primary antibodies used in this study are listed in Table 1. All antibodies have been extensively characterized for their specificity in previous studies (references in Table 1), using Western blotting, immunoprecipitation, as well as immunohistochemical staining of mice with targeted gene deletions ($GABA_A$ receptor α1, α3, and α5 subunit), and comparison with published studies (for the $GABA_A$ receptor α4 subunit, gephyrin, calcium-binding proteins and neuropeptides).

Tissue preparation

Perfusion-fixed tissue was prepared for immunoperoxidase and immunofluorescence staining in wildtype, GAD67-GFP, $\alpha1^{0/0}$ mice, and kainic acid-treated mice to analyze the regional distribution of either $GABA_A$ receptor subunits ($\alpha1$, $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$) or interneuron markers (PV, CR, CB, NPY, SOM) (Matyas *et al.*, 2004). Adult mice were deeply anesthetized with Nembutal (50 mg/kg, i.p.) and perfused through the ascending aorta with 4% paraformaldehyde in 0.15 M phosphate buffer (pH 7.4) containing 15% of a saturated picric acid solution. Brains were postfixed for 3 hours, cryoprotected in sucrose, frozen, and cut transversally at 40 μm with a sliding microtome. Sections were collected in PBS and stored in an antifreeze solution (15% glucose and 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) prior to use.

The subcellular distribution of the $\alpha1$, $\alpha2$ and $\alpha3$ subunits in CA1 interneurons from wildtype and $\alpha1^{0/0}$ mice was investigated by triple immunofluorescence staining in combination with gephyrin and a neurochemical marker. Optimal detection of neuropeptides and calcium-binding proteins usually is achieved in perfusion fixed tissue, whereas gephyrin and $GABA_A$ receptors are best detected in weakly fixed tissue (Fritschy *et al.*, 1998). For combined labeling, a novel protocol was developed (see Schneider Gasser *et al.* (2006) for details), which ensures preservation of soluble cytoplasmic proteins in weakly fixed tissue. In brief, brain slices were prepared as described below for electrophysiology. They were fixed by immersion for 10 min in 4% paraformaldehyde in 0.15 M phosphate buffer, washed with PBS, cryoprotected overnight in 30% sucrose in PBS and frozen on a flat surface in the cryostat. Parasagittal sections were cut at a thickness of 16 μm, mounted onto gelatin-coated glass slides, air-dried at room temperature for 30 seconds, and stored at -20°C for at least one hour.

Immunoperoxidase staining

Free-floating sections were incubated overnight at 4°C with primary antibodies (Table 1) diluted in Tris buffer containing 2% normal goat serum and 0.2% Triton X-100. Sections were then washed and incubated for 30 minutes at room temperature in biotinylated secondary antibodies (1:300; Jackson Immunoresearch, West Grove, PA) diluted 1:300 in Tris buffer containing 2% normal goat serum. After three washes, sections were incubated in the ABC complex (1:100 in Tris buffer) for 20 minutes (Vectastain Elite Kit; Vector

Laboratories, Burlingame, CA), washed again and reacted with diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) in Tris buffer (pH 7.7) containing 0.015% hydrogen peroxide. The color reaction was stopped after 5-15 minutes with ice-cold PBS. Sections were then mounted on gelatin-coated slides and air-dried. Finally, they were dehydrated with an ascending series of ethanol, cleared in xylene, and coverslipped with Eukitt (Erne Chemie, Dällikon, Switzerland). Sections from wildtype and mutant mice were processed in parallel under identical conditions to minimize variability in staining intensity.

Immunofluorescence staining

This technique was used to quantify interneuron markers in perfusion-fixed tissue and to analyze the subcellular distribution of $GABA_A$ receptor subunits and gephyrin in identified neurons. Sections were preincubated for one hour at room temperature in PBS containing 10% normal donkey serum and 0.2% Triton X-100, followed by overnight incubation at 4°C with one or a mixture of primary antibodies raised in different species (Table 1) diluted in the same solution. Sections were then washed extensively in PBS and incubated for 30 minutes at room temperature in corresponding affinity-purified donkey IgGs coupled to Cy3 (1:300), Cy2 (1:200) or Cy5 (1:200) (Jackson Immunoresearch). Sections were washed again, and coverslipped with aqueous mounting medium (Dako, Carpinteria, CA).

Image analysis

Sections processed for immunoperoxidase staining were analyzed with bright field microscopy (Axioskop; Zeiss, Jena, Germany). Differences in staining intensity and distribution were assessed visually in four animals per group. Digital photographs were taken with a high-resolution color camera (Axiocam; Zeiss). Identical acquisition parameters were used for wildtype and $\alpha1^{0/0}$ mice. Minimal adjustments of contrast and brightness were made to entire images if necessary. Sections processed for immunofluorescence staining for quantification of interneurons were visualized by epifluorescence microscopy (Zeiss Imager equipped with an Apotome module). The number of interneurons immunoreactive for PV, CR, CB, SOM or NPY in wildtype and $\alpha1^{0/0}$ mice was counted in the CA1 area bilaterally in four sections per mouse (n=3 per genotype). Using GAD67-GFP mice, the fraction of GAD67-positive interneurons in the

CA1 region that were double-labeled for the α1 subunit was assessed in a random sample of 100 interneurons from 3 sections per mouse (n = 3).

Triple immunofluorescence staining of $GABA_A$ receptor subunits and gephyrin in identified interneurons was visualized by confocal laser scanning microscopy (LSM-510 Meta; Zeiss) with a X100 Plan-Acromat objective (N.A. 1.4) and sequential acquisition of separate color channels. Image acquisition settings were adjusted to cover the entire dynamic range of the photomultipliers. The pinhole size was set to 1.0 Airy unit for each channel, and stacks of 10 consecutive sections (512 x 512) spaced by 0.3 µm were acquired at a magnification of 0.1 µm/pixel. For display, images were processed with the image-analysis program Imaris (Bitplane, Zurich, Switzerland). Images from three channels were overlaid and background was subtracted. The density of gephyrin- and $GABA_A$ receptor α subunit clusters was quantified from reconstructed volumes from 10 confocal images using the module "surpass". Individual clusters were identified as isolated objects by threshold segmentation and counted automatically. These data were averaged from ten images per animal (n = 3-6 mice per genotype). Statistical comparison was done using a non-parametric test.

Electrophysiology

Slice preparation

Mice from both sexes (P21-30) were anesthetized with isoflurane and decapitated. The brain was quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF, composition in mM: 125 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 11 glucose, oxygenated with 95% O_2 - 5% CO_2). The brain was affixed to a vibratome stage with cyanoacrylate and kept in the ice-cold ACSF for slicing. Parasagittal 300 to 350 µm-thick slices containing the hippocampus were prepared and incubated at 33°C for 20 min before being stored at room temperature (25°C) in oxygenated ACSF.

Electrophysiological recordings and analysis

Slices were transferred to a recording chamber and visualized with a CCD camera (PCO Vx45; Till Photonics, Munich, Germany) mounted on an upright microscope (BX51WI; Olympus, Tokyo, Japan), equipped with a long working distance water-immersion

objective (Xlumplan FI 20X, N.A. 0.95), a fourfold magnification changer, Nomarski-type differential interference contrast, and infrared illumination. Patch electrodes were pulled from borosilicate glass (GC150TC; Clark Instruments, Wilts, UK) and had an open tip resistance of 3–4 MΩ when filled with the internal solution. Kynurenic acid (2 mM) was added to the external ACSF solution to block excitatory synaptic transmission and CGP 55845 (1 μM, dissolved in DMSO) to block $GABA_B$ receptors. Recordings were made using a Multiclamp 700A patch-clamp amplifier (Axon Instruments, Molecular Devices, Sunnyvale, CA), filtered at 4 kHz for spontaneous events and 2 kHz for miniature events, digitized at 20 kHz, stored, and analyzed using IGOR Pro software (Wavemetrics, Lake Oswego, OR). Access resistance was monitored for all experiments, and cells were excluded from further analysis if it changed by >20% during the recording.

Whole-cell voltage-clamp recordings of sIPSCs from wildtype and $\alpha1^{0/0}$ stratum oriens interneurons were obtained at room temperature with a holding potential of –60 mV and a high chloride containing internal solution (in mM: 100 CsCl, 2 $MgCl_2$, 1 EGTA, 2 ATP, 0.3 GTP, and 40 HEPES, pH 7,2, 300 mOsm). Continuous recordings started after the holding current had stabilized and lasted 10-15 min for baseline period and for the same length of time in the presence of each applied drug. Diazepam (1 μM) was applied to test the affinity and degree of saturation of the receptors to benzodiazepine agonists in wildtype and $\alpha1^{0/0}$ mice. The amplitude of tonic current was assessed by applying picrotoxin (100 μM) to the bath. mIPSCs were recorded in the presence of TTX (0.5 μM). Inhibitory events were recorded five minutes after the application of each drug.

sIPSCs and mIPSCs were detected off-line automatically with the 'Mini Analysis' software (Synaptosoft; Decatur, GA) with the detection threshold set 5 times higher than the baseline noise level for spontaneous events and 3 times higher for miniature events. All detected events were counted for analysis of frequency. 200 events were randomly selected for each recorded cell. For analysis of kinetics and amplitude double events were rejected. All accepted IPSCs were aligned on the point of rise and the decay time constant was calculated by monoexponential fitting from 10 to 90% of the curve. For each condition at least five individual animals were used. Plots of cumulative probability were made for inter-interval event duration, amplitude and decay time constants of IPSCs from wildtype and $\alpha1^{0/0}$ mice. Statistical significance was assessed using unpaired Student's *t*-test from averaged values.

TABLE 1: ***List of primary antibodies used in the present study***

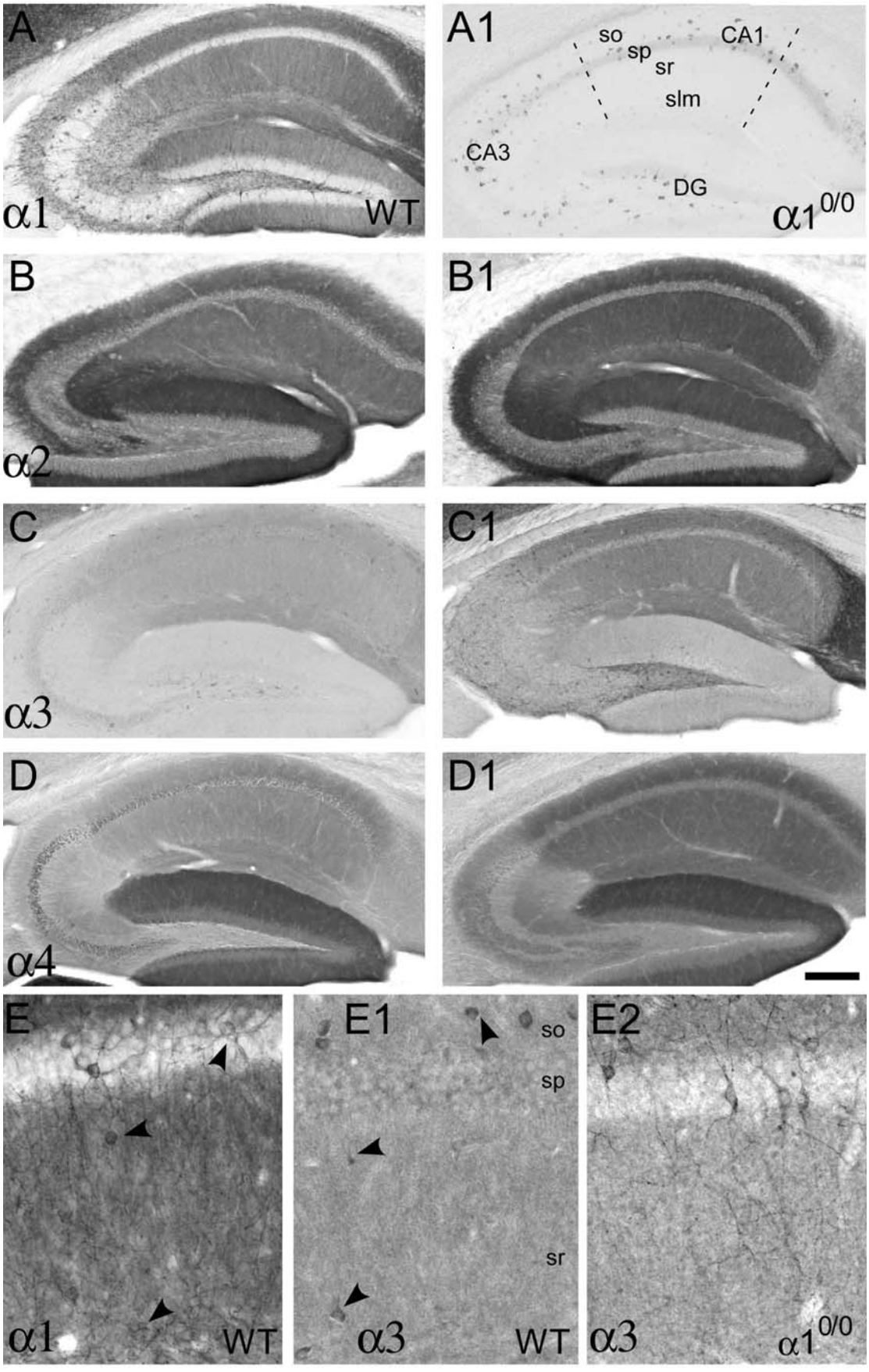
Target protein	Antigen	Species	Dilution Fixed tissue	Dilution slices	Source	References
$GABA_A$ receptor α1	rat N-terminal peptide 1-16	guinea pig serum	1:10000	1:50000	in house	(Fritschy & Mohler, 1995; Kralic *et al.*, 2006)
$GABA_A$ receptor α2	rat N-terminal peptide 1-9	guinea pig, affinity purified	1:1000	1:3000	in house	(Marksitzer *et al.*, 1993; Fritschy & Mohler, 1995)
$GABA_A$ receptor α3	rat N-terminal peptide 1-15	guinea pig serum	1:3000	1:5000	in house	(Fritschy & Mohler, 1995; Studer *et al.*, 2006)
$GABA_A$ receptor α4	rat N-terminal peptide 1-14	rabbit, affinity-purified	1:1000	-	Dr. W. Sieghart, Vienna	(Bencsits *et al.*, 1999; Ogris *et al.*, 2006)
$GABA_A$ receptor α5	rat N-terminal peptide 1-10	guinea pig serum	1:2500	-	in house	(Fritschy & Mohler, 1995; Fritschy *et al.*, 1997)
gephyrin	affinity-purified rat glycine receptors	mouse monoclonal antibody 7a	-	1:1000	Synaptic Systems, Göttingen, Germany; No. 147011	(Pfeiffer *et al.*, 1984; Sassoè-Pognetto *et al.*, 2000)
PV	purified bovine parvalbumin	rabbit IgG	1:10000	1:5000	Immunostar, Hudson WI; No. 24428	(Bouilleret *et al.*, 2000b)
CR	recombinant human calretinin	rabbit serum	1:5000	1:5000	Swant, Cat. No. 7699/4	(Schwaller *et al.*, 1993)
CB	bovine calbindin D28k	rabbit IgG	1:5000	1:5000	Immunostar; No. 24427	(Bouilleret *et al.*, 2000b)
NPY	synthetic porcine NPY	rabbit serum	1:3000	1:3000	Immunostar; No. 22940	(Bouilleret *et al.*, 2000b)
SOM	somatostatin 28 (Tyr^{1-14})	rabbit serum	1:3000	1:3000	Immunostar; No. 20089	(Bouilleret *et al.*, 2000b)

Results

$GABA_A$ receptor α2, α3 and α4 subunits are upregulated in dorsal hippocampus of adult $α1^{0/0}$mice

Deletion of the α1 subunit gene resulted in a complete loss of α1 subunit-immunoreactivity (-IR), except in the layer of pyramidal cells and in the cell body of some interneurons (Fig. 1A-A1), where the residual staining might reflect the presence of an N-terminal protein fragment recognized by the antibody. As reported previously (Kralic *et al.*, 2006), staining for the α2, α3 and α4 subunit was increased in the hippocampal formation of $α1^{0/0}$ mice, notably in CA1 compared to wildtype littermates (Fig. 1B-D1), whereas α5 subunit-IR remained unchanged (not shown). For the α2 subunit, no change in regional or cellular distribution was evident despite the enhanced IR, suggesting a global increase in protein abundance (Fig. 1B, E). The α3 subunit-IR, which faintly labels the CA1 area and a scattered subpopulation of interneuron somata in wildtype tissue (Fig. 1C, E1) was increased in interneurons and became prominent also in their dendrites in sections from mutant mice (Fig. 1C1, E2), apparently replacing the missing α1 subunit in these cells. Altogether, these observations suggest that the absence of α1-$GABA_A$ receptors from pyramidal cells and interneurons in the hippocampal formation is compensated for by increased density of other receptors contributing to both phasic (α2, α3) and tonic (α4) inhibition. This compensation is subunit-specific and occurs in both pyramidal cells and interneurons.

Figure 1. Comparative distribution of $GABA_A$ receptor subunits α1 (**A, A1, E**), α2 (**B, B1**), α3 (**C, C1, E1, E2**) and α4 (**D, D1**) in dorsal hippocampus of wildtype (WT) (A-E, E1) and $α1^{0/0}$ mice (A1-D1, E2), visualized by immunoperoxidase staining in transverse sections**.** The dashed lines in panel A1 delineate the borders of the region in CA1 where cells were sampled for quantification throughout this study. **A-A1**: The α1 subunit-IR disappears in the mutant mouse section, although a weak staining remains in the pyramidal cell layers and, mainly in interneurons, possibly denoting the presence of an N-terminal protein fragment recognized by the antibody. **B-D1**: Global increase in α2, α3 and α4 subunit staining in $α1^{0/0}$ mice, most prominent in the CA1 area. **E-E2**: Higher magnification photomicrographs illustrating the α1 subunit-IR in CA1 interneurons in wildtype mice and their weak labeling for the α3 subunit; arrowheads point to positive interneurons. In mutant, the α3 subunit-IR becomes very prominent in a subset of interneurons, apparently replacing the α1 subunit. DG: dentate gyrus; so: stratum oriens; sp: stratum pyramidale; sr: stratum radiatum; slm: stratum lacunosum-moleculare. Scale bar = 240 μm (applies to A-D) and 60 μm (applies to E).


A
α1
WT
A1
so
sp
sr
slm
CA1
CA3
DG
α1 0/0
B
α2
B1
C
α3
C1
D
α4
D1
E
α1
WT
E1
so
sp
sr
α3
WT
E2
α3
α1 0/0

Unaltered distribution of interneurons in dorsal hippocampus of $\alpha 1^{0/0}$ mice

Given the prominent expression of the α1 subunit in distinct subpopulations of interneurons, notably PV- and NPY-positive interneurons (Gao & Fritschy, 1994), we determined whether the neurochemical phenotype of five major interneuron subtypes was altered in the CA1 area of adult $\alpha 1^{0/0}$ mice. Four of these markers (PV, CR, NPY, and SOM) are present in various proportions of α1-subunit-positive interneurons in rat CA1 area (Gao & Fritschy, 1994) and have a similar distribution in mice (Matyas *et al.*, 2004). The fifth marker, CB, is present in interneurons apparently devoid of α1 subunit-IR (Gao & Fritschy, 1994) and was selected as a negative control. It should be noted that although these five markers allow an unambiguous identification of interneurons by immunofluorescence staining, none of them defines a single functional cell class (e.g., chandelier cell, OLM-cell) (Toledo-Rodriguez *et al.*, 2005; Baude *et al.*, 2006).

As illustrated in Figure 2, the staining pattern, regional and laminar distribution, and dendritic morphology of interneurons identified with these five markers was not altered in the hippocampal formation of $\alpha 1^{0/0}$ mice. Each marker exhibited the typical distribution seen in wildtype mice from different strains (see Bouilleret *et al.* (2000b) and Matyas *et al.* (2004) for comparison). Except CB, which labels dentate gyrus granule cells and CA1 pyramidal cells in addition to interneurons, all these markers were selectively detected in interneurons. A quantitative analysis revealed no difference the number of interneuron profiles in the CA1 area between wildtype and mutant mice for any of these markers (Fig. 2F; mean ± SD; n=3 per mice per genotype). These results indicate that deletion of the α1 subunit gene had no apparent effect on the expression of neurochemical markers or the distribution and morphology of interneurons in the hippocampal formation.

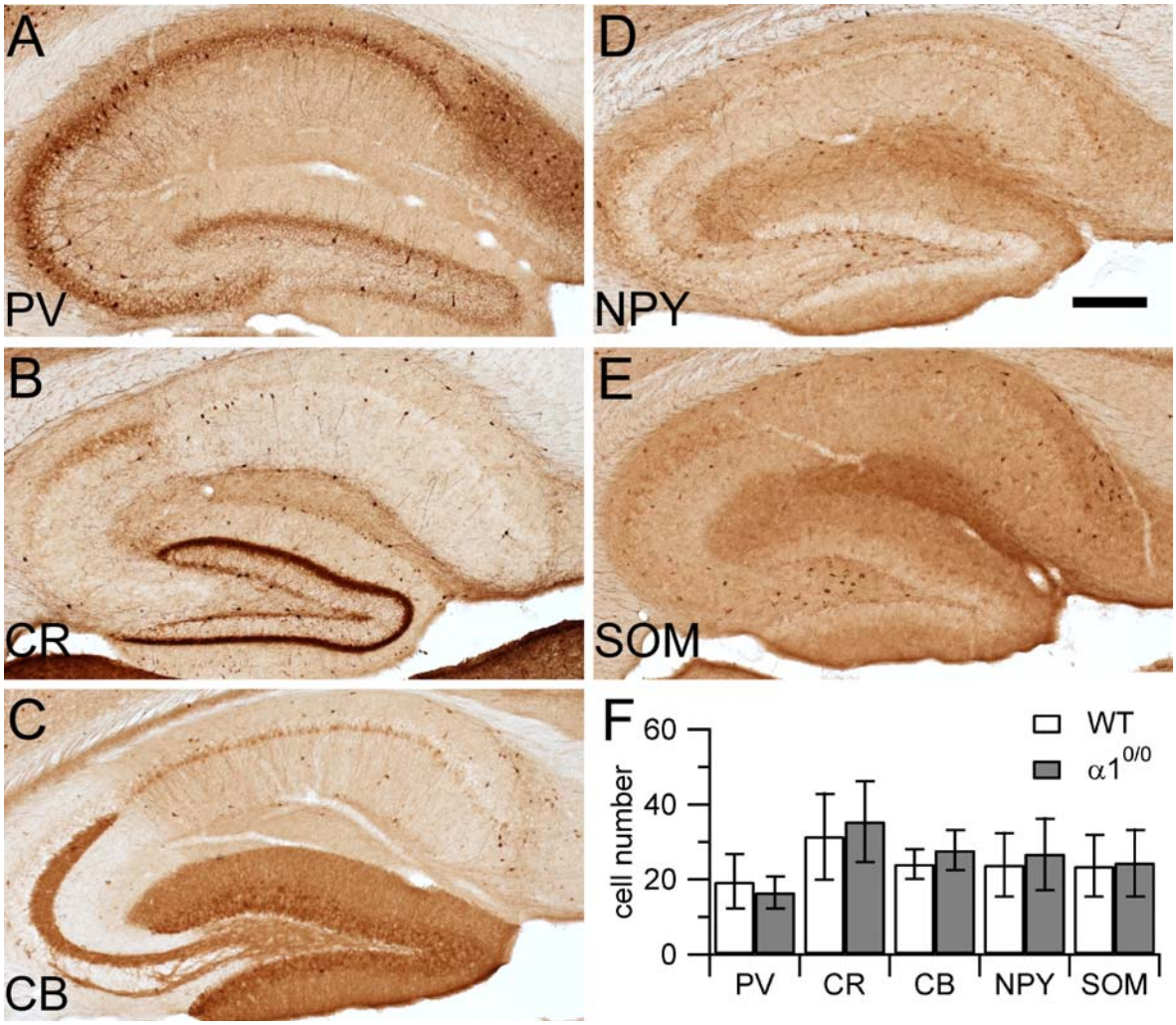


Figure 2. Distribution of PV- (**A**), CR- (**B**), CB- (**C**), NPY- (**D**) and SOM- (**E**) IR in dorsal hippocampus of $\alpha1^{0/0}$ mice, visualized by immunoperoxidase staining in transverse sections. Each of this marker exhibits a similar distribution pattern corresponding to that described in wildtype mice (Matyas *et al.*, 2004). In the CA1 area, PV-positive interneurons (**A**) are mostly located in stratum oriens (so) and stratum pyramidale (sp); CR- (**B**) and CB-positive interneurons (**C**) are located in all layers including stratum lacunosum-moleculare (slm); NPY-positive cells **(D)** are seen in the sp, so and stratum radiatum (sr) and SOM-positive cells (**E**) are only abundant in the so. **F**: The number of interneurons positive for each of these five markers remains unchanged between wildtype and $\alpha1^{0/0}$ mice (mean±SD; Student's t-test; n=3 mice/genotype). Scale bar = 240 μm.

Compensatory changes in $GABA_A$ receptor subunit-immunoreactivity in CA1 interneurons of $\alpha1^{0/0}$mice

First, to determine which fraction of interneurons express the α1 subunit in mouse CA1 area, immunofluorescence staining was performed in sections from GAD67-GFP mice (Tamamaki *et al.*, 2003), in which all interneurons are labeled with GFP. Random

sampling of CA1 interneurons in the dorsal hippocampus in three mice revealed 58-65% GFP-positive interneurons double-labeled for the α1 subunit, which outlined their soma and proximal dendrites. Most double-labeled cells were located in the stratum oriens, stratum pyramidale and lower half of stratum radiatum (data not shown).

Next, to determine whether the increased α2 and α3 subunit-IR in the CA1 region of $\alpha1^{0/0}$ mice contributes to compensate for the lack of α1-$GABA_A$ receptors in GABAergic synapses on interneurons, we performed a high resolution analysis of the distribution of the α1-α3 subunits along with gephyrin on the soma and proximal dendrites of interneurons identified by one of these five markers, using triple immunofluorescence staining. Gephyrin is selectively localized in the postsynaptic density of GABAergic synapses, as determined by pre- and postembedding immunoelectron microscopy (Sassoè-Pognetto *et al.*, 2000; Panzanelli *et al.*, 2004; Lagier *et al.*, 2007), and was taken here as a *bona fide* marker of GABAergic synapses. The present experiments were done in weakly fixed sections prepared from living slices to optimize the combined staining of the neurochemical marker with $GABA_A$ receptor subunit and gephyrin clusters at postsynaptic sites (Schneider Gasser *et al.*, 2006).

In wildtype mice, the α1 subunit-IR was detected in all subtypes of interneurons investigated, ranging from 100% in PV-positive cells to 33% CB-positive non-pyramidal cells (Table 2). In PV-, CR-, NPY- and SOM-immunoreactive cells, staining for the α1 subunit was mostly diffused around the soma (extrasynaptic) but a fraction was also clustered and colocalized with gephyrin, presumably representing synaptic receptors (Figs. 3A, 4A, 6A and 6E). CB-positive cells immunoreactive for the α1 subunit had only a small number of clusters double-labeled for the α1 subunit and gephyrin (Fig. 5A). Likewise, the majority of interneurons had a small number of α2 subunit-positive clusters, mainly on their dendrites, as seen by high resolution imaging in sections from wildtype mice (Fig. 3C, 4D, 5C-D; Table 2). An increase in α2-subunit-IR was detected in selected interneurons of $\alpha1^{0/0}$ mice, as detailed in the next section, although the proportion of interneurons carrying α2 subunit-positive clusters did not change significantly (Table 2). Finally, the α3 subunit-IR was undetectable by immunofluorescence in most CA1 interneurons from wildtype mice, except for a subpopulation located deep in the stratum oriens, representing a subset of PV-, NPY-, and SOM-positive cells (Table 2). In striking contrast, most interneurons from $\alpha1^{0/0}$ mice were positive for the α3 subunit, some of them having numerous clusters

around soma and dendrites. The genotype difference was significant in the five types of interneurons analyzed, and the proportion of α3 subunit-positive interneurons in $\alpha1^{0/0}$ mice in each subpopulation became highly similar to that seen for the α1 subunit in wildtype mice (Table 2). Therefore, $GABA_A$-mediated inhibition in CA1 interneurons is likely retained following α1 subunit gene deletion owing to upregulation of α3- and, to a lesser extent, α2-$GABA_A$ receptors. In contrast, the α4 and α5 subunit were not detectable in hippocampal interneurons from either wildtype or $\alpha1^{0/0}$ mice (not shown).

TABLE 2. ***Percentage of identified CA1 interneurons immunopositive for a given α subunit variant in wildtype (WT) and $\alpha1^{0/0}$ mice***

Interneuron marker	N (per genotype)	α1		α2		α3	
		WT	$\alpha1^{0/0}$	WT	$\alpha1^{0/0}$	WT	$\alpha1^{0/0}$
PV	6	100	0	96 ± 4	100	5 ± 4	98 ± 2**
CR	5	53 ± 5	0	69 ± 19	100	8 ± 8	52 ± 3**
CB	3	33 ± 8	0	83 ± 16	81 ± 19	2 ± 2	34 ± 9*
NPY	3	57 ± 4	0	58 ± 37	77 ± 20	7 ± 7	84 ± 8*
SOM	3	64 ± 6	0	68 ± 15	79 ± 15	12 ± 10	87±11*

Significant differences between average values in wildtype (WT) and $\alpha1^{0/0}$ mice are indicated (Mann-Whitney test, 2-tailed; *P<0.05; ** P<0.01).

Heterogeneity in cellular adaptation to the α1 subunit gene deletion

To further determine whether the compensatory changes occurring in $\alpha1^{0/0}$ mice affect the number of GABAergic postsynaptic sites and the subcellular distribution of $GABA_A$ receptors, the number of gephyrin and α subunit clusters located on labeled interneurons in wildtype and mutant mice was quantified in reconstructed volumes (51 x 51 x 3 μm) comprising the soma. The fraction of gephyrin clusters co-localizing with each α subunit was determined (Table 3), along with the ratio of gephyrin, α2 and α3 subunit clusters per cell between genotypes (Table 4). These data provide a quantitative assessment of the reorganization of inhibitory synapses on interneurons in response to the α1 subunit gene

deletion and demonstrate the dramatic upregulation of the α3 subunit in interneurons of mutant mice.

TABLE 3. ***Percentage of gephyrin clusters colocalized with α subunits in CA1 interneurons from wildtype (WT) and $\alpha1^{0/0}$ mice***

Interneuron marker	N (per genotype)	gephyrin/α1		gephyrin/α2		gephyrin/α3	
		WT	$\alpha1^{0/0}$	WT	$\alpha1^{0/0}$	WT	$\alpha1^{0/0}$
PV	6	83 ± 3	0	14 ± 2	30 ± 10	3 ± 1	70 ± 8**
CR	5	49 ± 12	0	34 ± 11	40 ± 15	17 ± 5	60 ± 7**
CB	3	36 ± 10	0	60 ± 24	58 ± 14	4 ± 1	42 ± 9*
NPY	3	66 ± 4	0	31 ± 18	30 ± 10	3 ± 2	70 ± 5*
SOM	3	70 ± 11	0	15 ± 3	13 ± 6	15 ± 5	67±22*

See Figures 3-6 for absolute values; Significant differences between average values in WT and $\alpha1^{0/0}$ mice are indicated (Mann-Whitney test, 2-tailed; *P<0.05; ** P<0.01).

TABLE 4: ***Ratio of postsynaptic cluster number in CA1 interneurons of $\alpha1^{0/0}$ and wildtype mice***

Interneuron type	gephyrin	α2	α3
PV	1.43*	4.56*	24.77**
CR	2.18*	1.81*	7.19**
CB	1.23	2.31*	39.94**
NPY	1.82*	1.07	38.39**
SOM	1.13	1.33	5.99**

See bar graphs in Figs. 3-6 for mean values (±SD). Significant differences between average values in WT and $\alpha1^{0/0}$ mice are indicated (Mann-Whitney test, 2-tailed; *P<0.05; ** P<0.01).

PV immunoreactive neurons

PV-IR neurons (Fig. 3) represent ~50% of all α1 subunit-positive neurons in the CA1 area and were all labeled for the α1 subunit, which outlined the soma and proximal dendrites and forms clusters co-localized with gephyrin (Fig. 3A). In addition, most PV-positive

interneurons have isolated α2 subunit-positive clusters, mainly on their dendrites (Fig 3C). The α3 subunit-IR, when detected, was located intracellularly (Fig. 3E). In $\alpha1^{0/0}$ mice, the density of α2 subunit-positive clusters was increased four-fold (Fig. 3D, 3G; Table 4), without change in subcellular localization. The increased α3 subunit-IR in PV-positive cells resulted in a 25-fold increase in the number of clusters colocalized with gephyrin (Fig. 3F-G; Table 4). The number of α3 subunit clusters in PV-positive interneurons from mutant mice was similar to the number of α1 subunit clusters counted on wildtype mice (Fig. 3G), but a decrease in extrasynaptic staining was evident. The total number of gephyrin clusters on PV-positive cells from mutant mice was increased by 43%, especially on proximal dendrites (Fig. 3B, G; Table 4), suggesting that GABAergic synaptic input on PV-positive cells is increased in $\alpha1^{0/0}$ mice.

CR immunoreactive neurons

CR-IR neurons (Fig. 4) represent ~25% of the α1 subunit positive neurons in the CA1 area. They form two distinct subpopulations distinguished by the presence or absence of dendritic spines. Most aspiny CR-IR cells were α1 subunit-positive (Figure 4A), whereas spiny CR-IR cells were mostly α1 subunit-negative (Fig. 4B). Isolated α2 subunit clusters were seen on most spiny (Fig 4D) and a fraction of aspiny CR-positive cell dendrites, whereas the α3 subunit-IR was rarely detected in either cell type (Fig. 3F, Table 4). In $\alpha1^{0/0}$ mice, the number of α2 subunit-positive clusters was increased almost 2-fold with unchanged cellular distribution (Fig. 4E, I; Table 4); likewise, the number of α3 subunit-positive clusters increased significantly (over 8-fold), notably in aspiny cells, covering the soma and proximal dendrites (Fig. 4G, I; Table 4). The increase in α3 subunit staining was also seen in spiny cells, but was less pronounced (Fig. 4H). These changes were accompanied by a 218% increase in the number of gephyrin clusters (Table 4), which, like in PV-subunit cells, were all colocalized with α2 or α3 subunit clusters (Table 3). Therefore, GABAergic synaptic coverage of CR-positive cells in $\alpha1^{0/0}$ mice was increased in aspiny cells, which normally express α1-$GABA_A$ receptors, but also in spiny cells, which appear largely devoid of gephyrin-labeled GABAergic synapses in wildtype mice.

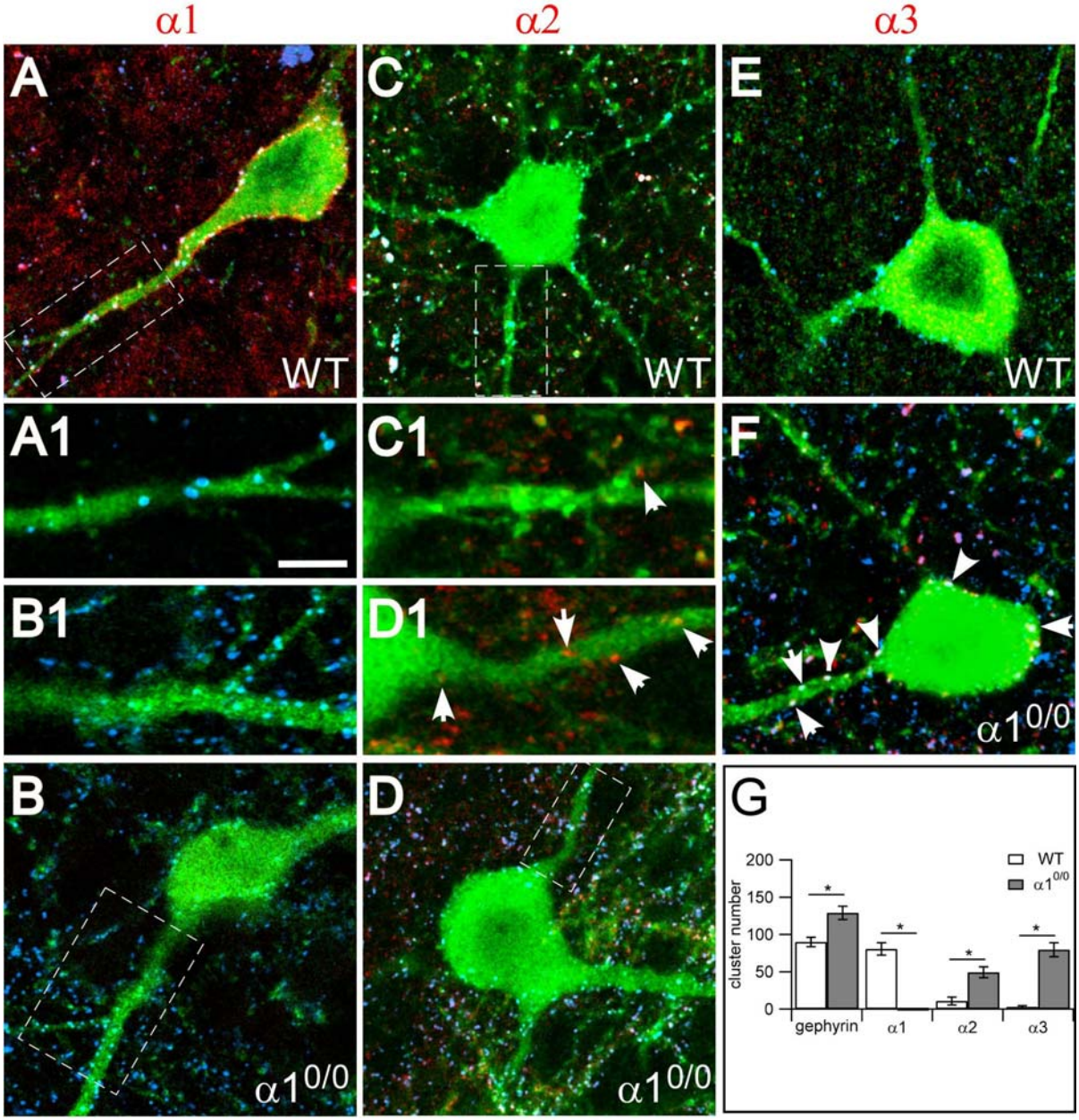


Figure 3. Comparative distribution of $GABA_A$ receptor α1, α2 and α3 subunit (red) and gephyrin (blue) clusters in PV-immunoreactive (green) CA1 interneurons of wildtype (WT) (**A, C, E**) and $\alpha1^{0/0}$ (**B, D, F**) mice, visualized by triple immunofluorescence staining and confocal laser scanning microscopy. All panels depict a single confocal section of cells located in the stratum oriens, close to the stratum pyramidale. **A**: In WT mice, the α1 subunit is located mostly extrasynaptically, outlining the soma and dendrites; clusters of α1 subunit-IR are few but colocalized with gephyrin (arrowheads). **B:** In $\alpha1^{0/0}$ mice an increase in the number of gephyrin clusters is observed. **A1-B1**: The difference in gephyrin clusters between WT and $\alpha1^{0/0}$ mice is better seen at high magnification in proximal dendrite segments (taken from the boxed area in panels A and B. **C-C1**: In WT mice, most PV-immunoreactive cells contain only isolated α2 subunit clusters on their soma and proximal dendrites (arrowhead in C1). **D-D1**: In $\alpha1^{0/0}$ mice an increased α2 subunit-IR is observed on some dendrites (arrowheads). Panels C1 and D1 are enlargement of the boxed areas in C and D. **E**: In WT mice, PV-positive cells are devoid of α3 subunit clusters. **F**: In $\alpha1^{0/0}$ mice, numerous α3 subunit clusters are evident on the soma and dendrites, colocalized with gephyrin (arrowheads). **G**: Quantification of gephyrin, α1, α2 and α3 subunit clusters revealed the significant increase of gephyrin, α2 and α3 subunit clusters in $\alpha1^{0/0}$ mice (n=6 mice/genotype). The vast majority of α2 and α3 subunits are colocalized with gephyrin (see table 4), indicating a postsynaptic localization. Scale bars: A-D, 10 μm; E-F, 10 μm.

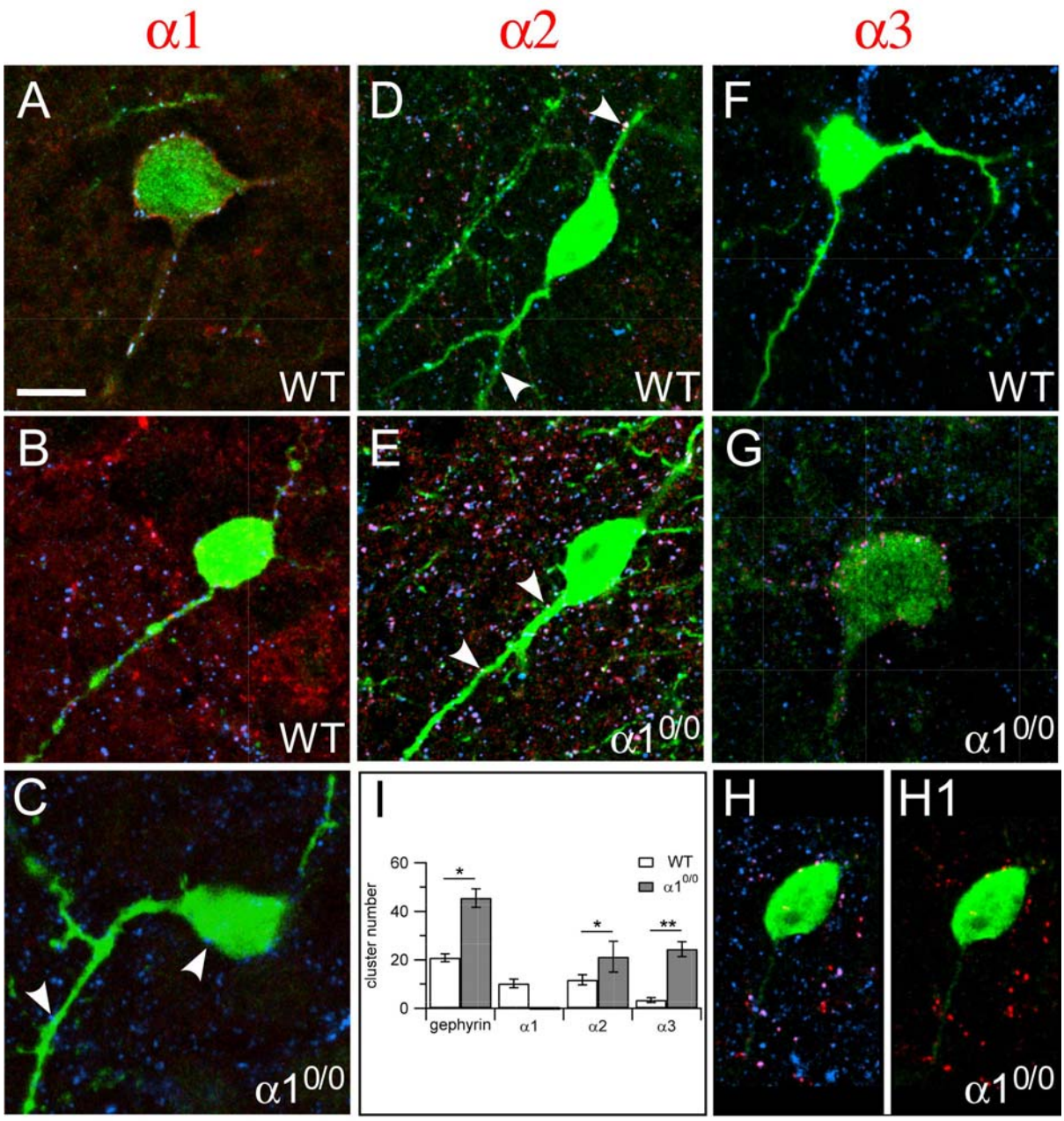


Figure 4. Comparative distribution of $GABA_A$ receptor α1, α2, α3 subunit (red) and gephyrin (blue) clusters in CR-immunoreactive (green) CA1 interneurons of wildtype (WT) (**A, B, D, F**) and $\alpha1^{0/0}$ (**C, E, G, H**) mice, visualized by triple immunofluorescence staining. All pictures are taken from cells located close to the stratum pyramidale. **A**: In WT mice, smooth, aspiny CR cells are positive for the α1 subunit, which forms only few clusters colocalized with gephyrin. **B**: Beaded, spiny CR cells appear devoid of α1 subunit-IR and have few gephyrin clusters on their soma and dendrites. **C**: In $\alpha1^{0/0}$ mice an increase in gephyrin clusters (arrowheads) occurs in both CR cell types, with a stronger increase in aspiny cells; this picture shows an example of a spiny CR cell. **D**: In wildtype mice, most CR cells present a small number of α2 subunit/gephyrin clusters on soma and dendrites (arrowheads). **E**: In $\alpha1^{0/0}$ mice α2 subunit clusters are increased in CR-positive cells (arrowheads). **F**: In WT mice, CR cells appear devoid of α3 subunit clusters. **G-H**: In $\alpha1^{0/0}$ mice α3 subunit clusters are found on the soma and dendrites of aspiny CR interneurons (**G**) and to a lesser extent in spiny CR cells (**H-H1**); the vast majority of α3 subunit clusters are colocalized with gephyrin. **J**: Quantification of gephyrin, α1, α2 and α3 subunit clusters revealed a significant increase of gephyrin, α2 and α3 clusters following deletion of the α1 subunit (n=5 mice/genotype). Scale bar: 10 μm (applies to all panels).

CB immunoreactive neurons

CB-positive interneurons in the CA1 area (Fig. 5) were distinguished from pyramidal cells by their size, multipolar cells bodies, and localization. In contrast to PV- and CR-immunoreactive neurons, α1 subunit-positive clusters were seen in only about 30% CB interneurons (Table 2). These clusters were located on the soma and dendrites and were colocalized with gephyrin; no diffuse (extrasynaptic) staining was evident (Fig. 5A). Although over 80% of CB interneurons were labeled for the α2 subunit in wildtype mice (Table 2), most of these cells carried only few α2 subunit clusters (Fig. 5C, J). However, a small subpopulation of CB-positive cells had a high density of postsynaptic α2 subunit clusters around their soma (Fig. 5D). These cells were located close to the pyramidal cell layer and might represent small "superficial" pyramidal cells, as described by (Baimbridge & Miller, 1982). In $\alpha1^{0/0}$ mice, a significant, more than 2-fold, increase in the number of α2 subunit-positive clusters colocalized with gephyrin was observed on soma and dendrites of CB-IR cells (Fig 5E, I; Table 4). Finally, very few CB interneurons carried α3 subunit-positive clusters (Fig. 5F, I) in wildtype mice. In mutant, however, a high density of α3 subunit clusters was seen in about 30% of CB-positive cells in mutant mice (Table 2), of which only about 20% were colocalized with gephyrin (Fig. 5G-I). It was not possible to determine whether these interneurons correspond to those expressing α1-$GABA_A$ receptors in wildtype mice. Despite the increase in α2 subunit clusters, the overall density of gephyrin clusters was not increased significantly in CB-positive cells of $\alpha1^{0/0}$ mice (Fig. 5B, I), but they were frequently colocalized with either α2 or the α3 subunit clusters (Table 3).

These results suggest that CB-positive interneurons undergo differential compensatory changes in $\alpha1^{0/0}$ mice, although many of them do not normally express the α1 subunit. In most CB-interneurons, GABAergic synaptic input is preserved by upregulation of postsynaptic α2-$GABA_A$ receptors and, in a subset of cells, mainly extrasynaptic α3-$GABA_A$ receptors (or postsynaptic receptors not associated with gephyrin) are formed in mutant mice. GABAergic regulation of CB interneurons is, therefore, different in wildtype and $\alpha1^{0/0}$ mice.

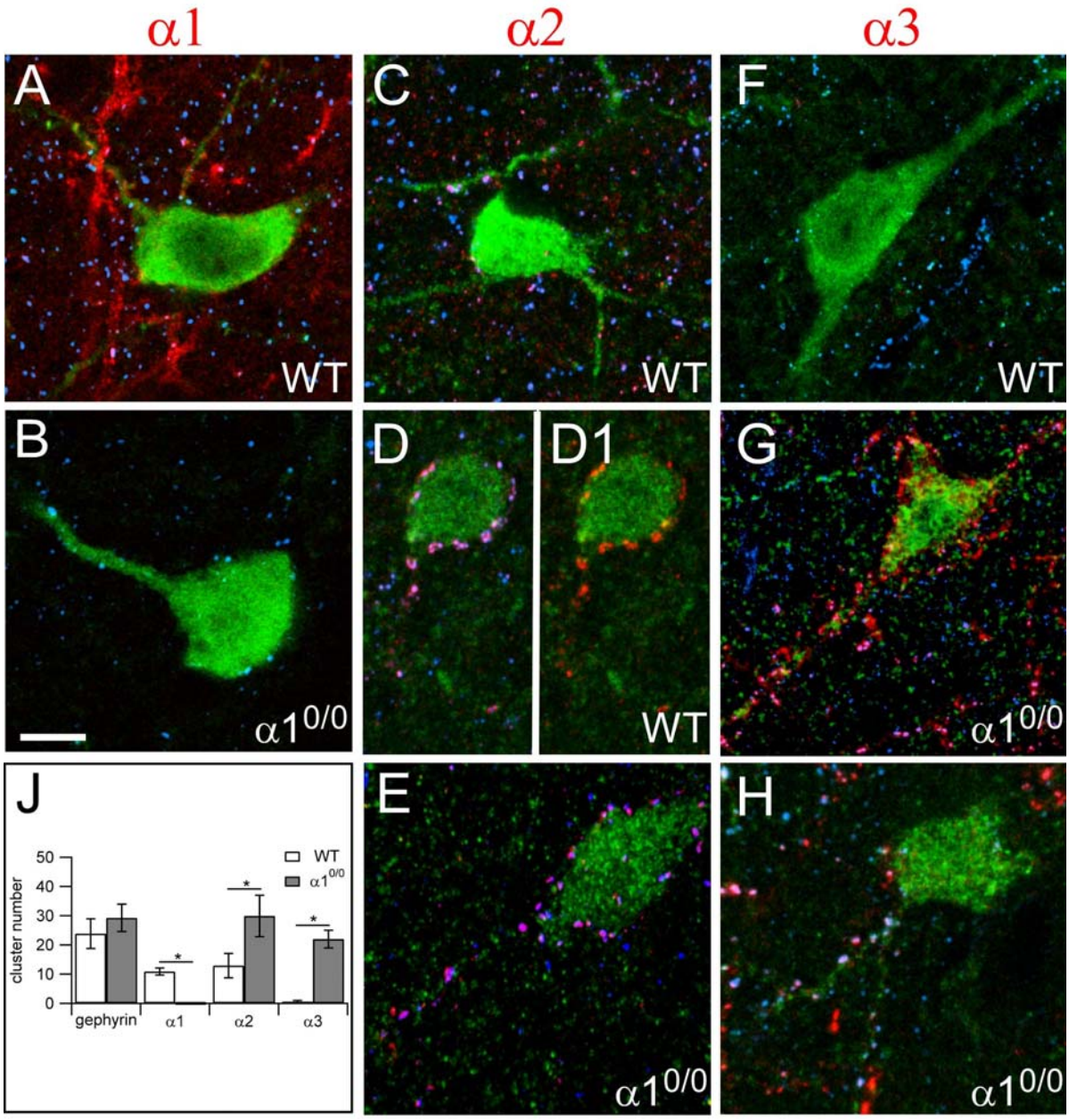


Figure 5. Comparative distribution of $GABA_A$ receptor α1, α2, α3 subunit (red) and gephyrin (blue) clusters in CB-immunoreactive (green) CA1 interneurons of wildtype (WT) (**A, C, D, F**) and $\alpha1^{0/0}$ (**B, E, G, H**) mice. Pictures were taken from cells located in stratum radiatum. **A**: In WT mice, a third of CB interneurons carry α1 subunit-positive clusters (arrowheads) partially colocalized with gephyrin; in this picture, the dendrites of CB-negative cells are strongly positive for the α1 subunit. **B**: In $\alpha1^{0/0}$ mice no upregulation of gephyrin clusters occurs. **C-D**: In WT mice, the α2 subunit is either weakly detectable on dendrites of CB cells (**C;** arrowhead) or abundantly on soma (**D-D1**), always clustered and colocalized with gephyrin. **E**: An upregulation of the α2 subunit-IR, clustered and colocalized with gephyrin, is seen in CB-positive interneurons of $\alpha1^{0/0}$ mice. **F**: In WT mice, CB-positive interneurons are devoid of α3 subunit clusters. **G-H**: In $\alpha1^{0/0}$ mice, most CB-positive interneurons exhibit α3 subunit clusters on soma and dendrites, colocalized with gephyrin in about one third of these cells (compare **G** and **H**). **J**: Quantification of gephyrin, α1, α2 and α3 clusters reveals a significant increase of α2 and α3 subunit clusters, but not gephyrin clusters, following deletion of the α1 subunit (n=3 mice/genotype). The scale bar in A represents 10 μm in A-F and G-H and 7.5 μm in D-D1.

NPY and SOM immunoreactive neurons

NPY- and SOM-positive interneurons (Fig. 6) represent ~30% and ~10% of α1 subunit-positive interneurons in the CA1 area, respectively. For both subtypes, 60% of interneurons were found to be positive for the α1 subunit (Fig. 6A, D, E, H; Table 2). Few α2 or α3 subunit clusters were detected in these cell types (Fig. 6D, H). In $\alpha1^{0/0}$ mice, no upregulation of α2 subunit was evident; however, numerous α3 subunit-positive clusters were seen, extensively colocalized with gephyrin (Fig 6C-D, G-H; Table 3). The number of gephyrin clusters was significantly increased in NPY-, but not SOM-positive interneurons in mutant mice (Fig 6B, F; Table 4).

These observations suggest that only α3-$GABA_A$ receptors are upregulated in NPY- and, to a lesser extent, SOM-positive interneurons in $\alpha1^{0/0}$ mice. In both cell types, most of these receptors are associated with gephyrin and, therefore, postsynaptic.

In summary $\alpha1^{0/0}$ mice exhibited an increase of gephyrin clusters in PV-, CR- and NPY-IR cells and retained the number of gephyrin clusters in CB- and SOM- IR cells. A general increase of α3 subunit clusters was evident in all cell types and, in addition, of α2 subunit clusters in PV-, CR- and CB-IR interneurons. The α2 subunit was always colocalized with gephyrin whereas the α3 subunit formed clusters that were sometimes devoid of gephyrin, especially in CB-IR cells.

Phasic GABAergic transmission is partially impaired in CA1 interneurons of $\alpha1^{0/0}$mice

To determine whether $GABA_A$ receptor-mediated phasic and tonic inhibition is retained in interneurons of $\alpha1^{0/0}$ mice, we performed whole-cell voltage-clamp recordings of sIPSCs and mIPSCs from CA1 stratum oriens interneurons. The cells to be recorded were identified visually by their bright somata and dendritic arborization.

sIPSCs were seen in all interneurons recorded from both genotypes (Fig. 7A, B). Their mean frequency did not differ between wildtype and $\alpha1^{0/0}$ mice (3.6 ± 0.5 and 3.3 ± 0.5 Hz, respectively; Fig. 7D; Table 5). However, their mean amplitude was slightly, non-significantly reduced (Fig. 7C, E) whereas their mean decay time was prolonged by 62% (Fig. 7C, F; Table 5) in mutant mice. Application of 100 μM picrotoxin to the bath solution blocked all sIPSCs, indicating that they were $GABA_A$ receptor-mediated. The change in holding current after application of picrotoxin (Fig. 7A-B, right trace) was equal between

wildtype and $\alpha1^{0/0}$ mice interneurons (21.5 pA and 22.1 pA, respectively), indicating that tonic inhibition was preserved.

Application of diazepam (1 μM), which interacts indiscriminately with α1-, α2-, α3- and α5-$GABA_A$ receptors, increased sIPSC frequency, amplitude and decay time in both wildtype and $\alpha1^{0/0}$ mice. Amplitude and decay time were increased in the same proportion in both genotypes (~15% increase in amplitude and ~30% increase in decay time constant) whereas the increase in sIPSC frequency after diazepam application was ~37% larger in $\alpha1^{0/0}$ than in wildtype mice (data not shown).

Recordings of mIPSCs revealed a significant decrease in mean frequency in interneurons of $\alpha1^{0/0}$ mice (Fig. 8A-B, D) (2.6 ± 0.5 and 1.0 ± 0.5, respectively; Table 5), which contrasts with the increased number of gephyrin clusters reported above. The mean mIPSC amplitude was also decreased by ~25% in $\alpha1^{0/0}$ mice compared to wildtype (Fig. 8C, E), while the mean decay time constant increased by 112% (Fig. 8C, F; Table 5). All mIPSCs were blocked with picrotoxin (not shown), indicating that they were $GABA_A$ receptor-mediated.

TABLE 5. ***Characteristics of sIPSC and mIPSC in CA1 interneurons***

	Number of cells	Frequency, Hz	Amplitude, pA	Decay time constant, ms
sIPSCs				
WT	6	3.6 ± 3.6	-43.6 ± 13.5	10.8 ± 5.0
$\alpha1^{0/0}$	6	3.3 ± 3.3	-37.7 ± 8.2	17.5 ± 3.6**
% change from WT		-6.7	-13.4	62
mIPSCs				
WT	9	2.6 ± 2.6	-36.9 ± 4.2	8.5 ± 1.4
$\alpha1^{0/0}$	6	1.0 ± 1.3**	-27.7 ± 9.8*	18.1 ± 6.9*
% change from WT		-61	-24.8	112.9

Significant differences between average values in WT and $\alpha1^{0/0}$ mice are indicated (Mann-Whitney test, 2-tailed; *P<0.05; ** P<0.01).

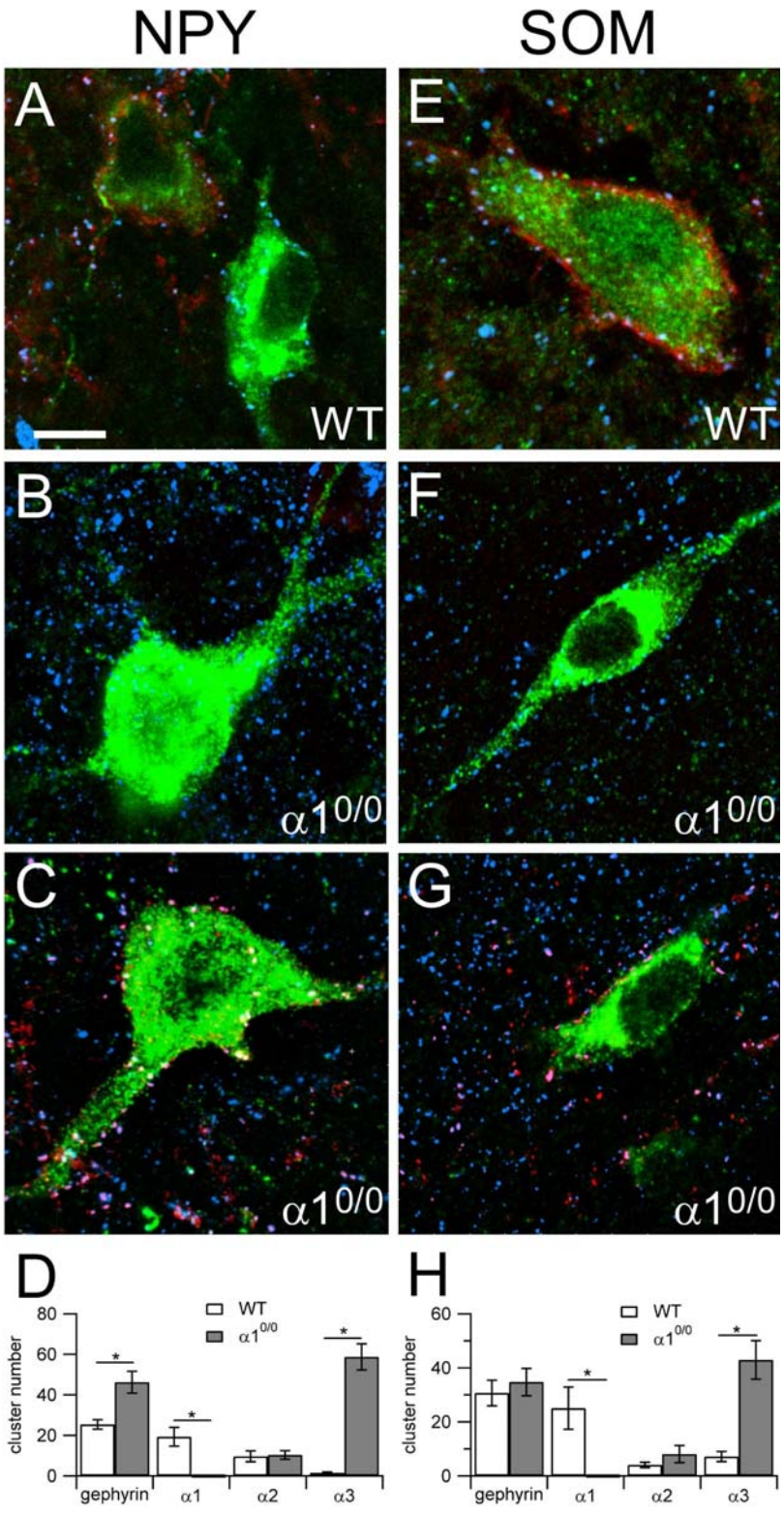


Figure 6.
Comparative distribution of $GABA_A$ receptor α1 and α3 subunit (red) and gephyrin (blue) clusters in NPY- and SOM-immunoreactive CA1 interneurons (green) of wildtype (WT) (**A, E**) and $\alpha1^{0/0}$ (**B, C, F, G**) mice, visualized by triple immunofluorescence staining. Pictures were taken from cells located in the stratum oriens. In WT mice the α1 subunit, detected in 57% NPY-positive cells (**A**) and 64% SOM-positive cells (**E**), is prominent but forms few clusters co-localized with gephyrin. **B**, **F**: In $\alpha1^{0/0}$ mice an increase in the number of gephyrin clusters is evident in NPY cells (**B**) but not in SOM cells (**F**). **C, G**: In both cell types numerous α3 subunit clusters colocalized with gephyrin are apparent in $\alpha1^{0/0}$ mice. **D, H**: Quantification of gephyrin, α1, α2 and α3 clusters reveals a significant increase of gephyrin in NPY cells and α3 clusters in both cell types following deletion of the α1 subunit (n=3 mice/genotype). The scale bar in A represent 10 μm in the left column and 8 μm in the right column.

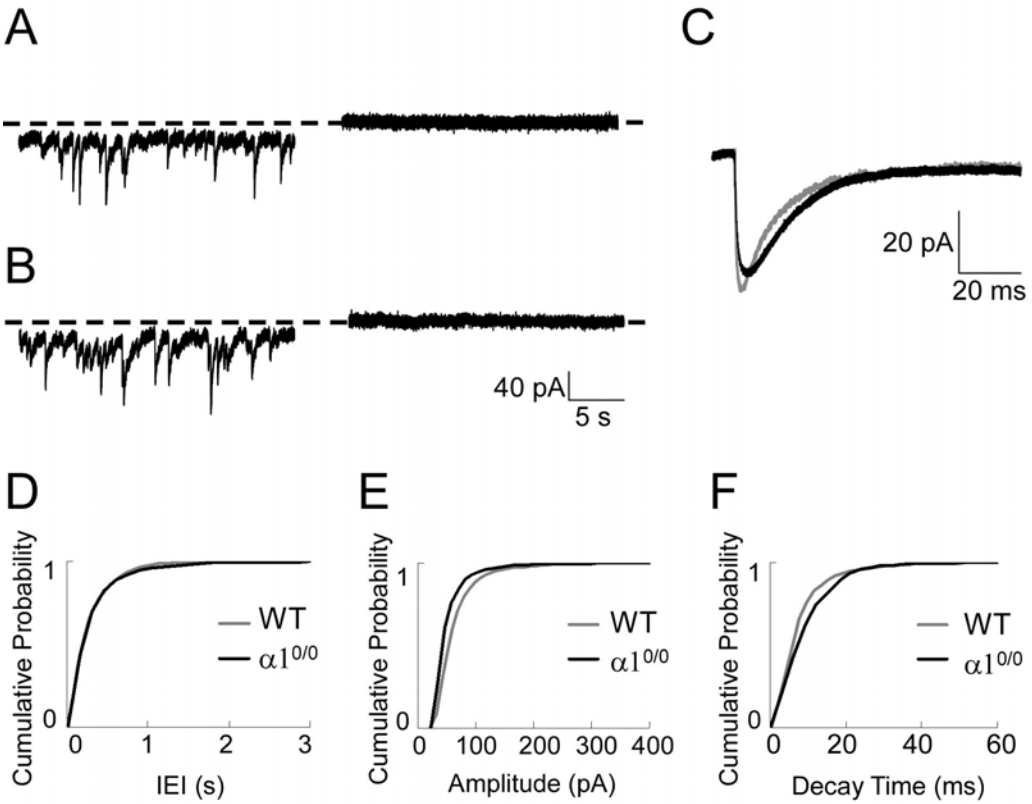


Figure 7. Spontaneous inhibitory postsynaptic currents (sIPSCs) in CA1 stratum oriens interneurons from wildtype (WT) and $\alpha1^{0/0}$ mice. **A-B:** Representative traces of sIPSCs recorded in an interneuron from a WT (**A**) and $\alpha1^{0/0}$ mouse (**B**), in the absence (left) and presence of 100 μM (right) picrotoxin. The inward change in holding current induced by picrotoxin, denoting tonic inhibition, had the same amplitude in both genotypes (21.5 pA and 22.1 pA, respectively). **C:** Averaged traces from the interneurons in A (grey) and B (black). Note the small reduction in amplitude and the increase in decay time occurring in $\alpha1^{0/0}$ mice. **D:** Cumulative probability distribution of sIPSC inter-event-intervals (IEI) in WT and $\alpha1^{0/0}$ mice; no change in frequency is evident between genotypes. **E:** Cumulative probability distribution of sIPSC amplitude, which is not significantly different in $\alpha1^{0/0}$ mice ($P<0.3$). **F:** cumulative probability distribution of sIPSC decay time constants, showing the significant increase in $\alpha1^{0/0}$ mice ($P<0.02$).

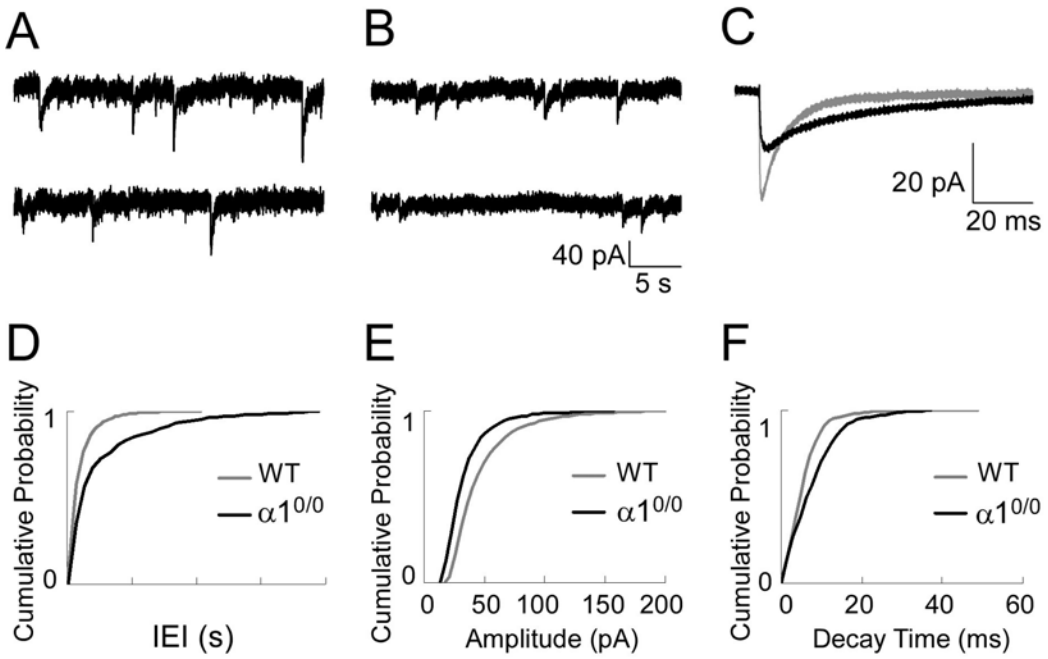


Figure 8. Miniature inhibitory postsynaptic currents (mIPSCs) in stratum oriens CA1 interneurons from wildtype (WT) and $\alpha1^{0/0}$ mice. **A-B:** Representative traces of mIPSCs recorded in WT (A) and $\alpha1^{0/0}$ mice (B). **C:** Averaged traces from the interneurons in A (grey trace) and B (black trace). Note the significant reduction in amplitude and the increase in decay time in $\alpha1^{0/0}$ mice. **D-F:** Cumulative probability distribution of mIPSC inter-event-intervals (IEI; **D**), amplitude (**E**), and decay time constants (**F**), showing a significant reduction in the frequency ($P<0.001$) and amplitude ($P<0.03$), but an increase in decay time constant ($P<0.02$) in $\alpha1^{0/0}$ mice.

Wildtype and $\alpha1^{0/0}$mice have the same sensitivity to kainic acid-induced lesions

To determine whether the compensatory changes in the organization of $GABA_A$ receptor subtypes are functionally relevant *in vivo*, we investigated whether the acute and chronic excitotoxic action of kainic acid is altered in $\alpha1^{0/0}$ mice. To this end, adult mice received a unilateral intrahippocampal injection of kainic acid and were examined histologically after 14 days post-treatment. This model was selected because it is extensively characterized, with kainic acid injection causing temporally distinct morphological alterations (Bouilleret *et al.*, 2000a). Acutely, it causes degeneration of CA1 and CA3 pyramidal cells, along with loss of hilar cells and a large subset of interneurons in CA1 and the dentate gyrus (mostly affecting PV-positive cells); chronically, it induces on-going degeneration of most

pyramidal cells in CA1 and CA3 and a pronounced hypertrophy and dispersion of dentate gyrus granule cells, which is dependent on BDNF-induced signaling (Suzuki *et al.*, 1995). Upon kainic acid injection, mice experience a non-convulsive status epilepticus, followed by a silent phase prior to onset of spontaneous recurrent focal seizures (Riban *et al.*, 2002). This model therefore allows monitoring distinct effects of kainic acid along with sensitivity to convulsions and epileptogenesis.

No difference in severity or duration of status epilepticus was observed in $\alpha1^{0/0}$ mice compared to wildtype upon kainic acid injection. Examination of Nissl-stained sections 14 days post-treatment revealed no different in lesion size or granule cell dispersion between genotypes (not shown). Finally, immunohistochemistry for interneuron markers revealed no difference in their susceptibility to kainate-induced toxicity (Fig. 9; compare to Fig. 2). Therefore, the compensatory changes occurring in $\alpha1^{0/0}$ mice allow a "wildtype-like" response to kainic acid injection. Furthermore, replacing α1-$GABA_A$ receptors in PV-positive interneurons by α2- and α3-$GABA_A$ receptors, which have distinct functional properties, does not make them more resistant to kainic acid, despite the slower kinetics of the corresponding receptors.

Discussion

The present study reveals extensive rearrangements of $GABA_A$ receptors and GABAergic circuits to compensate for the absence of the α1 subunit in CA1 interneurons. Unlike Purkinje cells, which remain devoid of functional $GABA_A$ receptors in $\alpha1^{0/0}$ mice and lose a majority of GABAergic synapses on their dendrites (Fritschy *et al.*, 2006), the density of gephyrin clusters, denoting postsynaptic sites, increases in CA1 interneurons, along with clusters of α3, and to a lesser extent, α2 subunit. This effect is cell-specific, since the density of gephyrin clusters remains unchanged in CB- and SOM-positive interneurons.

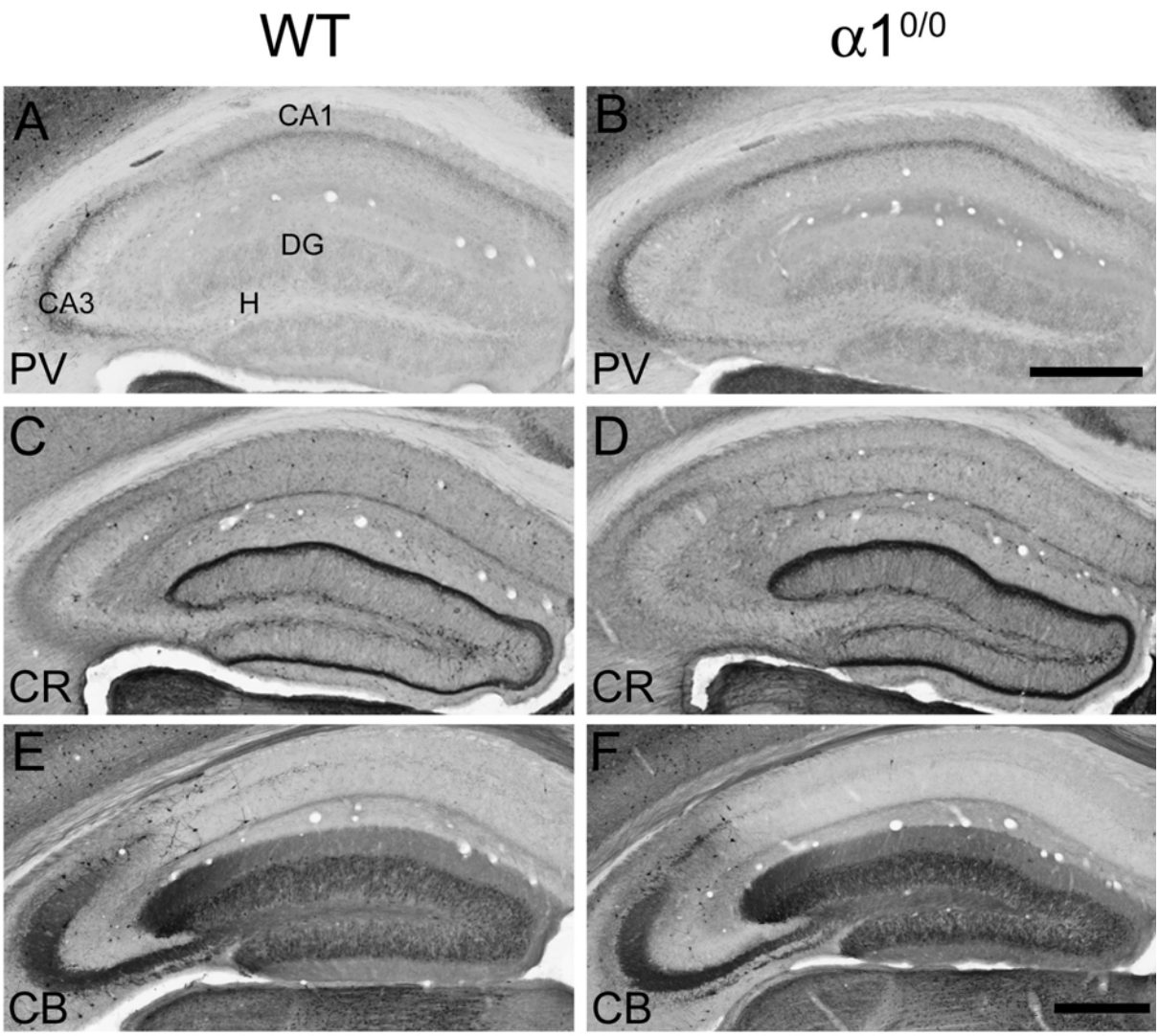


Figure 9. Similar alterations in the distribution of interneuron markers in kainic acid-treated wildtype (WT) and $\alpha1^{0/0}$ mice, as seen in the injected hippocampal formation 14 days after treatment. At this time-point, dispersion of dentate gyrus (DG) granule cells causes an enlargement of this structure. In CA1 and CA3, degeneration of pyramidal cells is only partial. **A-B**: Almost complete loss of PV-IR in CA1 and DG is evident in both genotypes. Compare to Fig. 2 for the distribution pattern seen in control mice. **C-D**: The majority of CR-positive cells are retained in CA1 and CA3, along with the prominent innervation of the inner molecular layer of the DG. **E-G**: The majority of CB-positive interneurons are lost in CA1, along with a decreased staining of pyramidal cells. In contrast, CB-IR appears increased in DG granule cells and mossy fibers and is largely normal in CA3 in both genotypes. H, hilus. Scale bar: A-D, 200 μm; E-F, 150 μm.

Although previous studies had suggested that α1 subunit-positive CA1 interneurons express no other α subunit variant (Gao & Fritschy, 1994; Fritschy & Mohler, 1995; Pirker *et al.*, 2000), we demonstrate here an unsuspected heterogeneity of $GABA_A$ receptors in these cells, with a majority of CR, CB, NPY, and SOM interneurons expressing a small number of α2 subunits-positive clusters, along with the α1 and/or α3 subunit. Even most PV interneurons, which predominantly express α1-$GABA_A$ receptors, also had a few α2 subunit clusters on their dendrites. Thus, the detection of subunit clusters colocalized with gephyrin on identified interneurons in weakly fixed tissue (Schneider Gasser *et al.*, 2006) provides improved sensitivity compared to the use of perfusion-fixed tissue. Strikingly, deletion of the α1 subunit gene caused a stronger increase in α3 than α2 subunit clusters. This observation suggests that the missing α1 subunit in mutant mice is not randomly replaced by another $GABA_A$ receptor subtype, but that the compensatory upregulation occurs in defined neuronal circuits. Several other findings support this conclusion: 1) the increase in α2 and α3 subunit clusters even occurs in cells that normally do not express the α1 subunit, such as most CB-positive interneurons; 2) the subcellular distribution of $GABA_A$ receptors in $\alpha1^{0/0}$ mice is distinct from that seen in wildtype mice, notably due to a decrease in extrasynaptic staining and an increase in perisomatic clusters; 3) the increase in gephyrin clusters suggests formation of additional GABAergic synapses, although an upregulation from undetectable levels in pre-existing synapses cannot formally be ruled out. These morphological rearrangements were not sufficient to fully restore the function of α1-$GABA_A$ receptors, since the amplitude and frequency of mIPSPs were significantly reduced in interneurons from $\alpha1^{0/0}$ mice, in line with previous results (Goldstein *et al.*, 2002). However, on the network level, GABAergic inhibition appears to be functional, since $\alpha1^{0/0}$ mice are not more susceptible than wildtype mice to the acute and chronic excitotoxic and epileptogenic action of kainic acid.

The major effect of the α1 subunit gene deletion is a loss of about 50% $GABA_A$ receptors in the brain (Sur *et al.*, 2001; Kralic *et al.*, 2002). Despite pronounced increase in protein content for several α subunit variants, the loss of α1 subunit is paralleled by a decrease in β2 and γ2 subunit (Kralic *et al.*, 2006; Ogris *et al.*, 2006), indicating that the α1 subunit is required for assembly of this major $GABA_A$ receptor subtype. All previous studies of $\alpha1^{0/0}$ mice have shown that the upregulation of $GABA_A$ receptor subunits does not involve a change in gene expression (Sur *et al.*, 2001; Bosman *et al.*, 2005; Ogris *et al.*, 2006),

suggesting increased translation and/or reduced turnover of other $GABA_A$ receptor subtypes. Studying the thalamus and cerebellum, we have shown that α1-$GABA_A$ receptors are not "replaced" by these compensatory changes (Kralic *et al.*, 2006). Rather, the α3 and α4 subunits are increased, without change in cellular or subcellular distribution. An extreme situation was seen in cerebellum, where Purkinje cells are devoid of functional $GABA_A$ receptors despite an 8-fold increase in α3 subunit clusters in the same tissue. These observations implied a change in GABAergic circuits and formation of novel synapses containing the up-regulated receptors. A more complex situation occurs in cells that co-express the α1 and α3 subunit. Thus, in olfactory bulb mitral cells of $\alpha1^{0/0}$ mice, the loss of α1-$GABA_A$ receptors leads to a partial compensation by α3-$GABA_A$ receptors, mainly in GABAergic synapses formed on the soma; however, the number of functional synapses is reduced in mitral cell dendrites, with slower decay kinetics and lower amplitude of sIPSCs (Lagier *et al.*, 2007), as observed here. In turn, these altered functional properties have major consequences on the network level, resulting in decreased frequency of γ oscillations induced by olfactory nerve stimulation (Lagier *et al.*, 2007). These findings underscore the importance of GABAergic synapses for coordinated function of neuronal networks and demonstrate that each $GABA_A$ receptor subtype confers unique functional properties to brain circuits.

The contrast between the very weak expression of the α3 subunit in CA1 interneurons (Fig. 1), except for a specific subpopulation in stratum oriens (Brünig *et al.*, 2002), and the strong immunoreactivity seen in $\alpha1^{0/0}$ mice underscores the plasticity of this $GABA_A$ receptor subtype. Our quantitative analysis suggests that the α3 subunit "replaces" the missing α1 subunit, notably in PV- and NPY-positive interneurons. "De novo" expression of the α3 subunit appears unlikely. Rather, upregulation from low constitutive levels might be the mechanism of this striking compensatory change. It is of note that the number of α2 and α3 subunit clusters in CB- and CR-positive interneurons of mutant mice largely exceeds the number of α1 subunit clusters in these cells in wildtype, indicating that the upregulation of these subunits is not a mere compensation, but reflects a reorganization of GABAergic circuits in the hippocampus, as had been noted previously in the cerebellum (Kralic *et al.*, 2006).

Aside from the prolongation in decay time constant, little effect of the mutation was seen in recordings of sIPSCs in interneurons from wildtype and $\alpha1^{0/0}$ mice. Since the recorded

interneurons were not identified morphologically, it is not excluded that potential changes affecting only a specific class might have been overlooked. However, the prolongation of decay matches the general up-regulation of the α3 subunit in most interneurons in mutant mice. In line with the morphological results, $GABA_A$ receptors in interneurons remain sensitive to diazepam, as shown by an increased frequency and prolongation of sIPSCs in the presence of this drug. Also, tonic $GABA_A$-mediated inhibition was not altered by the mutation, confirming that GABAergic transmission is largely retained in mutants. The latter result was surprising, since tonic inhibition in interneurons is likely mediated by $GABA_A$ receptors containing the δ subunit, along with α1 (Glykys *et al.*, 2007). Since we failed to detect α4 and α5 subunit-IR in interneurons, the question remains open whether the δ subunit forms receptors with either α2 or α3 in mutant mice.

The amplitude and decay time constant values of mIPSCs recorded here in stratum oriens interneurons are different from those reported for interneurons in stratum radiatum and stratum lacunosum-moleculare (Goldstein *et al.*, 2002), suggesting either differences in recording conditions or in intrinsic properties of interneurons. However, the differences between genotypes were proportionally similar, and the values for mIPSC frequency were nearly identical between the two studies. Using non-stationary fluctuation analysis upon application of zolpidem, Goldstein et al. (2002) observed a reduction in the number of $GABA_A$ receptors open at the peak of mIPSCs, which they suggested explains the lower amplitude and frequency of mIPSCs in $\alpha1^{0/0}$ mice. However, the marked reduction in mIPSC amplitude might be due to the lower GABA affinity of α3-$GABA_A$ receptor (Ebert *et al.*, 1994; Verdoorn, 1994), rather than a reduction in the number of receptors in the synapse. The lower frequency of mIPSC might, in part, be secondary to their decreased amplitude but could also reflect a presynaptic effect. Since our morphological data point towards an increase in GABAergic synaptic coverage of interneurons, as reflected by the density of gephyrin clusters, a presynaptic alteration might include decreased presynaptic GABA release, due to partial filling of presynaptic vesicles, or to changes in GABA clearance in the synapse (Barberis *et al.*, 2004). Such a phenomenon has been demonstrated to occur in GABAergic synapses from cultured neurons upon prolonged reduction of neuronal activity (Hartman *et al.*, 2006). The 37% larger effect of diazepam on the frequency of mIPSPs in interneurons from mutant than wildtype mice (not shown) would also be compatible with a difference in $GABA_A$ receptor saturation between genotypes. Therefore, the possibility of a presynaptic deficit in transmitter release or

vesicular storage would be compatible with both morphological and functional data. However, such a deficit remains to be demonstrated and would imply that the absence of α1-$GABA_A$ receptors indirectly influences the maturation or function of presynaptic terminals.

GABAergic input to CA1 interneurons is derived from intrinsic and extrinsic sources. Intrinsically, CR-positive interneurons and to a lesser extent CB and SOM interneurons innervate other interneurons (Seress *et al.*, 1993; Freund & Gulyas, 1997; Gulyas *et al.*, 1999; Matyas *et al.*, 2004). PV interneurons are also known to innervate each other (Meyer *et al.*, 2002; Bartos *et al.*, 2002), likely to maintain synchronized activity. Extrinsically, medial septal GABAergic cells projecting to the hippocampus selectively target interneurons (Freund & Antal, 1988; Freund & Gulyas, 1997). CR- and CB-positive cells receive fewer GABAergic synapses than PV interneurons (Gulyas *et al.*, 1999), in line with the distribution of gephyrin clusters reported here (Fig. 3G, 4J, 5J). Assuming that a deficit in transmitter release or vesicular storage in the presynaptic GABAergic afferents of CA1 interneurons underlies the decreased mIPSCs seen in $\alpha1^{0/0}$ mice, the effect might occur either secondarily to a change in activity (Hartman *et al.*, 2006) of either local CR-positive cells or septohippocampal neurons or it might reflect a deregulation of presynaptic control of transmitter release by a G-protein coupled receptor.

The results obtained in kainic acid-treated mice provide two important cues about the function and regulation of hippocampal interneurons. First, they show that the differential vulnerability of interneurons subtypes to kainic acid (Bouilleret *et al.*, 2000a, b) is not related to the expression of a specific $GABA_A$ receptor subtype, since replacing α1-$GABA_A$ receptors by α3-$GABA_A$ receptors did not protect PV interneurons against kainic acid-induced acute degeneration. Secondly, they demonstrate that the functional compensations to the absence of the α1 subunit are operative and sufficient to produce stable neuronal networks that can resist kainic acid-induced excitation without becoming epileptic. In this mouse model, kainic acid injection induces a spreading wave of neuronal firing that is contained by synaptic inhibition, and leads secondarily to interictal activities (Le Duigou *et al.*, 2005). The "normal" response of $\alpha1^{0/0}$ mice to kainic acid-induced overexcitation strongly suggests that GABAergic inhibition is operative in the hippocampal formation of these mutants.

Altogether, the present results demonstrate a striking plasticity of GABAergic circuits in the hippocampal formation, and suggest that changes in inhibitory control of interneurons underlie the compensatory adaptations that occur to maintain the function of hippocampal networks in the absence of a major $GABA_A$ receptor subtype. Since $GABA_A$ receptors expressed in $\alpha1^{0/0}$ neurons have different functional properties, the plasticity of GABAergic circuits goes beyond receptor replacement and likely includes formation of novel synaptic connections. These findings are of immediate relevance for the pathophysiology of temporal lobe epilepsy, in which GABAergic synapses remain active, but have novel properties due to extensive functional and morphological reorganization of GABAergic networks (Cossart *et al.*, 2005). On a broader perspective, it is to be expected that compensatory changes that ensure stable function of neuronal networks in $\alpha1^{0/0}$ mice are not limited to the GABAergic system, but comprise a broad array of homeostatic mechanisms designed to maintain network integrity after deletion of one key component (Marder & Goaillard, 2006).

Acknowledgments

We wish to thank to Dr. Greg Homanics (University of Pittsburgh) for providing us the $\alpha1^{0/0}$ mice, Dr. Y. Yanagawa (Gunma University Graduate School of Medicine) for GAD67-GFP mice, Dr. Werner Sieghart (University of Vienna) for antibodies against the $\alpha4$ subunit, Corinne Sidler and Franziska Parpan for excellent technical assistance and extensive mouse genotyping work and Kaspar Vogt for advice with electrophysiology. The work was supported by the Swiss National Science Foundation.

List of Abbreviations

CB, calbindin

CR, calretinin

-IR, -immunoreactivity

mIPSC, miniature inhibitory postsynaptic current

NPY, neuropeptide Y

PV, parvalbumin

SOM, somatostatin

sIPSC, spontaneous inhibitory postsynaptic current

References

Baimbridge, K.G. & Miller, J.J. (1982) Immunohistochemical localization of calcium-binding protein in the cerebellum, hippocampal formation and olfactory bulb of the rat. *Brain Res.*, 245, 223-229.

Barberis, A., Lu, C., Vicini, S. & Mozrzymas, J.W. (2005) Developmental changes of GABA synaptic transient in cerebellar granule cells. *Mol. Pharmacol.*, 67, 1221-1228.

Barberis, A., Petrini, E.M. & Cherubini, E. (2004) Presynaptic source of quantal size variability at GABAergic synapses in rat hippocampal neurons in culture. *Eur. J. Neurosci.*, 20, 1803-1810.

Barnard, E.A. (2001) The molecular architecture of $GABA_A$ receptors. In Mohler, H. (ed.) *Pharmacology of GABA and glycine neurotransmission*. Springer-Verlag, Berlin, pp. 79-100.

Bartos, M., Vida, I., Frotscher, M., Meyer, A.H., Monyer, H., Geiger, J.R. & Jonas, P. (2002) Fast synaptic inhibition promotes synchronized γ oscillations in hippocampal interneuron networks. *Proc. Natl. Acad. Sci. USA*, 99, 13222-13227.

Baude, A., Bleasdale, C., Dalezios, Y., Somogyi, P. & Klausberger, T. (2006) Immunoreactivity for the $GABA_A$ receptor α1 subunit, somatostatin and connexin36 distinguishes axoaxonic, basket, and bistratified interneurons of the rat hippocampus. *Cereb. Cortex.*, Nov 30; [Epub ahead of print].

Bencsits, E., Ebert, V., Tretter, V. & Sieghart, W. (1999) A significant part of native γ-aminobutyric $acid_A$ receptors containing α4 subunits do not contain γ or δ subunits. *J. Biol. Chem.*, 274, 19613-19616.

Benke, D., Fritschy, J.M., Trzeciak, A., Bannwarth, W. & Mohler, H. (1994) Distribution, prevalence and drug-binding profile of $GABA_A$-receptors subtypes differing in β-subunit isoform. *J. Biol. Chem.*, 269, 27100-27107.

Bosman, L.W.J., Heinen, K., Spijker, S. & Brussaard, A.B. (2005) Mice lacking the major adult $GABA_A$ receptor subtype have normal number of synapses, but retain juvenile IPSC kinetics until adulthood. *J. Neurophysiol.*, 94, 338-346.

Bosman, L.W.J., Rosahl, T.W. & Brussaard, A.B. (2002) Neonatal development of the rat visual cortex: synaptic function of $GABA_A$ receptor α subunits. *J. Physiol.*, 545, 169-181.

Bouilleret, V., Loup, F., Kiener, T., Marescaux, C. & Fritschy, J.M. (2000a) Early loss of interneurons and delayed subunit-specific changes in $GABA_A$-receptor expression in a mouse model of mesial temporal lobe epilepsy. *Hippocampus*, 10, 305-324.

Bouilleret, V., Ridoux, V., Depaulis, A., Marescaux, C., Nehlig, A. & Le Gal La Salle, G. (1999) Recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult mice: EEG, histopathology and synaptic reorganization similar to mesial temporal lobe epilepsy. *Neuroscience*, 89, 717-729.

Bouilleret, V., Schwaller, B., Schurmans, S., Celio, M.R. & Fritschy, J.M. (2000b) Neurodegenerative and morphogenic changes in a mouse model of temporal lobe epilepsy do not depend on the expression of the calcium-binding proteins parvalbumin, calbindin, or calretinin. *Neuroscience*, 97, 47-58.

Brünig, I., Scotti, E., Sidler, C. & Fritschy, J.M. (2002) Intact sorting, targeting, and clustering of γ-aminobutyric acid A receptor subtypes in hippocampal neurons *in vitro*. *J. Comp. Neurol.*, 443, 43-45.

Cossart, R., Bernard, C. & Ben-Ari, Y. (2005) Multiple facets of GABAergic neurons and synapses: multiple fates of GABA signalling in epilepsies. *Trends Neurosci.*, 28, 108-115.

Ebert, B., Wafford, K.A., Whiting, P.J., Krogsgaard-Larsen, P. & Kemp, J.A. (1994) Molecular pharmacology of γ-aminobutyric acid type A receptor agonists and partial agonists in oocytes injected with different α, β, and γ receptor subunit combinations. *Mol. Pharmacol.*, 46, 957-963.

Freund, T.F. & Antal, M. (1988) GABA-containing neurons in the septum control inhibitory interneurons in the hippocampus. *Nature*, 336, 170-173.

Freund, T.F. & Gulyas, A.I. (1997) Inhibitory control of GABAergic interneurons in the hippocampus. *Can. J. Physiol. Pharmacol.*, 75, 479-487.

Fritschy, J.M., Benke, D., Johnson, D.K., Mohler, H. & Rudolph, U. (1997) $GABA_A$-receptor α-subunit is an essential prerequisite for receptor formation *in vivo*. *Neuroscience*, 81, 1043-1053.

Fritschy, J.M. & Mohler, H. (1995) $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J. Comp. Neurol.*, 359, 154-194.

Fritschy, J.M., Panzanelli, P., Kralic, J.E., Vogt, K.E. & Sassoè-Pognetto, M. (2006) Differential dependence of axo-dendritic and axo-somatic GABAergic synapses on

$GABA_A$ receptors containing the α1 subunit in Purkinje cells. *J. Neurosci.*, 26, 3245-3255.

Fritschy, J.M., Weinmann, O., Wenzel, A. & Benke, D. (1998) Synapse-specific localization of NMDA- and $GABA_A$-receptor subunits revealed by antigen-retrieval immunohistochemistry. *J. Comp. Neurol.*, 390, 194-210.

Gao, B. & Fritschy, J.M. (1994) Selective allocation of $GABA_A$-receptors containing the α1-subunit to neurochemically distinct subpopulations of hippocampal interneurons. *Eur. J. Neurosci.*, 6, 837-853.

Glykys, J., Peng, Z., Chandra, D., Homanics, G.E., Houser, C.R. & Mody, I. (2007) A new naturally occurring $GABA_A$ receptor subunit partnership with high sensitivity to ethanol. *Nature Neurosci.*, 10, 40-48.

Goldstein, P.A., Elsen, F.P., Ying, S.W., Ferguson, C., Homanics, G.E. & Harrison, N.L. (2002) Prolongation of hippocampal miniature inhibitory postsynaptic currents in mice lacking the $GABA_A$ receptor α1 subunit. *J. Neurophysiol.*, 88, 3208-3217.

Gulyas, A.I., Hajos, N., Katona, I. & Freund, T.F. (2003) Interneurons are the local targets of hippocampal inhibitory cells which project to the medial septum. *Eur. J. Neurosci.*, 17, 1861-1872.

Gulyas, A.I., Megias, M., Emri, Z. & Freund, T.F. (1999) Total number and ratio of excitatory and inhibitory synapses converging onto single interneurons of different types in the CA1 area of the rat hippocampus. *J. Neurosci.*, 19, 10082-10097.

Hartman, K., N., Pal, S.K., Burrone, J. & Murthy, V.N. (2006) Activity-dependent regulation of inhibitory synaptic transmission in hippocampal neurons. *Nature Neurosci.*, 9, 642-649.

Hutcheon, B., Morley, P. & Poulter, M.O. (2000) Developmental change in $GABA_A$ receptor desensitization kinetics and its role in synapse function in rat cortical neurons. *J. Physiol.*, 522, 3-17.

Klausberger, T., Marton, L., O'Neill, J., Huck, J., Dalezios, Y., Fuentealba, P., Suen, W.Y., Papp, E., Kaneko, T., Watanabe, M., Csicsvari, J. & Somogyi, P. (2005) Complementary roles of cholecystokinin- and parvalbumin-expressing GABAergic neurons in hippocampal network oscillations. *J. Neurosci.*, 25, 9782-9793.

Kralic, J.E., Criswell, H.E., Ostermann, J.L., O'Buckley, T.K., Wilkie, M.E., Matthews, D.A., Hamre, K., Breese, G.R., Homanics, G.E. & Morrow, A.L. (2005a) Genetic

essential tremor in γ-aminobutyric acid$_A$ receptor α1 subunit knock-out mice. *J. Clin. Invest.*, 115, 774-779.

Kralic, J.E., Ledergerber, D.A. & Fritschy, J.M. (2005b) Disruption of the neurogenic potential of the dentate gyrus in a mouse model of temporal lobe epilepsy with focal seizures. *Eur. J. Neurosci.*, 22, 1916-1927.

Kralic, J.E., O'Buckley, T.K., Khisti, R.T., Hodge, C.J., Homanics, G.E. & Morrow, A.L. (2002) GABA$_A$ receptor α1 subunit deletion alters receptor subtype assembly, pharmacological and behavioral responses to benzodiazepines and zolpidem. *Neuropharmacol.*, 43, 685-694.

Kralic, J.E., Sidler, C., Parpan, F., Homanics, G., Morrow, A.L. & Fritschy, J.M. (2006) Compensatory alteration of inhibitory synaptic circuits in thalamus and cerebellum of GABA$_A$ receptor α1 subunit knock-out mice. *J. Comp. Neurol.*, 495, 408-421.

Lagier, S., Panzanelli, P., Russo, R.E., Nissant, A., Bathellier, B., Sassoè-Pognetto, M., Fritschy, J.M. & Lledo, P.M. (2007) GABAergic inhibition at dendrodendritic synapses tunes γ oscillations in the olfactory bulb. *Proc. Natl. Acad. Sci. USA*, in press.

Le Duigou, C., Wittner, L., Danglot, L. & Miles, R. (2005) Effects of focal injection of kainic acid into the mouse hippocampus *in vitro* and ex vivo. *J. Physiol.*, 569, 833-847.

Marder, E. & Goaillard, J.M. (2006) Variability, compensation and homeostasis in neuron and network function. *Nature Rev. Neurosci.*, 7, 563-574.

Marksitzer, R., Benke, D., Fritschy, J.M. & Mohler, H. (1993) GABA$_A$-receptors: Drug binding profile and distribution of receptors containing the α2-subunit in situ. *J. Recept. Res.*, 13, 467-477.

Matyas, F., Freund, T.F. & Gulyas, A.I. (2004) Immunocytochemically defined interneuron populations in the hippocampus of mouse strains used in transgenic technology. *Hippocampus*, 14, 460-481.

Matyas, F., Freund, T.F. & Gulyas, A.I. (2004) Convergence of excitatory and inhibitory inputs onto CCK-containing basket cells in the CA1 area of the rat hippocampus. *Eur. J. Neuroscience*, 19, 1243-1256.

McKernan, R.M., Rosahl, T.W., Reynolds, D.S., Sur, C., Wafford, K.A., Atack, J.R., Farrar, S., Myers, J., Cook, G., Ferris, P., Garrett, L., Bristow, L., Marshall, G., Macaulay, A., Brown, N., Howell, O., Moore, K.W., Carling, R.W., Street, L.J., Castro, J.L., Ragan, C.I., Dawson, G.R. & Whiting, P.J. (2000) Sedative but not

anxiolytic properties of benzodiazepines are mediated by the $GABA_A$ receptor α1 subtype. *Nature Neurosci.*, 3, 587-592.

Meyer, A.H., Katona, I., Blatow, M., Rozov, A. & Monyer, H. (2002) *In vivo* labeling of parvalbumin-positive interneurons and analysis of electrical coupling in identified neurons. *J. Neurosci.*, 22, 7055-7064.

Mohler, H., Fritschy, J.M. & Rudolph, U. (2002) A new benzodiazepine pharmacology. *J. Pharm. Exp. Ther.*, 300, 2-8.

Nusser, Z., Sieghart, W., Benke, D., Fritschy, J.M. & Somogyi, P. (1996) Differential synaptic localization of two major γ-aminobutyric acid type A receptor α subunits on hippocampal pyramidal neurons. *Proc. Natl. Acad. Sci. USA*, 93, 11939-11944.

Ogris, W., Lehner, R., Fuchs, K., Furtmuller, B., Hoger, H., Homanics, G.E. & Sieghart, W. (2006) Investigation of the abundance and subunit composition of $GABA_A$ receptor subtypes in the cerebellum of α1-subunit-deficient mice. *J. Neurochem.*, 96, 136-147.

Panzanelli, P., Homanics, G.E., Ottersen, O.P., Fritschy, J.M. & Sassoè-Pognetto, M. (2004) Pre- and postsynaptic $GABA_A$ receptors at reciprocal dendrodendritic synapses in the olfactory bulb. *Eur. J. Neurosci.*, 20, 2945-2952.

Persohn, E., Malherbe, P. & Richards, J.G. (1992) Comparative molecular neuroanatomy of cloned $GABA_A$ receptor subunits in the rat CNS. *J. Comp. Neurol.*, 326, 193-216.

Pfeiffer, F., Simler, R., Grenningloh, G. & Betz, H. (1984) Monoclonal antibodies and peptide mapping reveal structural similarities between the subunits of the glycine receptor of rat spinal cord. *Proc. Natl. Acad. Sci. USA*, 81, 7224-7227.

Pirker, S., Schwarzer, C., Wieselthaler, A., Sieghart, W. & Sperk, G. (2000) $GABA_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience*, 101, 815-850.

Prenosil, G.A., Schneider Gasser, E.M., Rudolph, U., Keist, R., Fritschy, J.M. & Vogt, K.E. (2006) Specific subtypes of $GABA_A$ receptors mediate phasic and tonic forms of inhibition in hippocampal pyramidal neurons. *J. Neurophysiol.*, 96, 846-857.

Riban, V., Bouilleret, V., Pham-Lè, B.T., Fritschy, J.M., Marescaux, C. & Depaulis, A. (2002) Evolution of hippocampal epileptic activity during the development of hippocampal sclerosis in a mouse model of temporal lobe epilepsy. *Neuroscience*, 112, 101-111.

Rudolph, U., Crestani, F., Benke, D., Brünig, I., Benson, J., Fritschy, J.M., Martin, J.R., Bluethmann, H. & Mohler, H. (1999) Benzodiazepine actions mediated by specific γ-aminobutyric acid$_A$ receptor subtypes. *Nature*, 401, 796-800.

Rudolph, U., Crestani, F. & Mohler, H. (2001) GABA$_A$ receptor subtypes: dissecting their pharmacological functions. *Trends Pharmacol. Sci.*, 22, 188-194.

Sassoè-Pognetto, M. & Fritschy, J.M. (2000) Gephyrin, a major postsynaptic protein of GABAergic synapses. *Eur. J. Neurosci.*, 7, 2205-2210.

Sassoè-Pognetto, M., Panzanelli, P., Sieghart, W. & Fritschy, J.M. (2000) Co-localization of multiple GABA$_A$ receptor subtypes with gephyrin at postsynaptic sites. *J. Comp. Neurol.*, 420, 481-498.

Schneider Gasser, E.M., Straub, C.J., Panzanelli, P., Weinmann, O., Sassoè-Pognetto, M. & Fritschy, J.M. (2006) Immunofluorescence in brain sections: simultaneous detection of presynaptic and postsynaptic proteins in identified neurons. *Nature Protocols*, 1, 1887-1897.

Schwaller, B., Buchwald, P., Blumcke, I., Celio, M.R. & Hunziker, W. (1993) Characterization of a polyclonal antiserum against the purified human recombinant calcium binding protein calretinin. *Cell Calcium*, 14, 639-648.

Seress, L., Gulyas, A.I., Ferrer, I., Tunon, T., Soriano, E. & Freund, T.F. (1993) Distribution, morphological features, and synaptic connections of parvalbumin- and calbindin D28K-immunoreactive neurons in the human hippocampal formation. *J. Comp. Neurol.*, 337, 208-230.

Sieghart, W. & Ernst, M. (2005) Heterogeneity of GABA$_A$ receptors: revived interest in the development of subtype-selective drugs. *Curr. Med. Chem.*, 5, 217-242.

Studer, R., von Boehmer, L., Haenggi, T., Schweizer, C., Benke, D., Rudolph, U. & Fritschy, J.M. (2006) Alteration of GABAergic synapses and gephyrin clusters in the thalamic reticular nucleus of GABA$_A$ receptor α3 subunit-null mice. *Eur. J. Neurosci.*, 24, 1307-1315.

Sur, C., Wafford, K.a., Reynolds, D.S., Hadingham, K.L., Bromidge, F., Macaulay, A., Collinson, N., O'Meara, G., Howell, O., Newman, R., Myers, J., Atack, J.R., Dawson, G.R., McKernan, R.M., Whiting, P.J. & Rosahl, T.W. (2001) Loss of the major GABA$_A$ receptor subtype in the brain is not lethal in mice. *J. Neurosci.*, 21, 3409-3418.

Suzuki, F., Junier, M.P., Guilhem, D., Sorensen, J.C. & Onteniente, B. (1995) Morphogenetic effect of kainate on adult hippocampal neurons associated with a prolonged expression of brain-derived neurotrophic factor. *Neuroscience*, 64, 665-674.

Tamamaki, N., Yanagawa, Y., Tomioka, R., Miyazaki, J., Obata, K. & Kaneko, T. (2003) Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. *J. Comp. Neurol.*, 467, 60–79.

Toledo-Rodriguez, M., Goodman, P., Illic, M., Wu, C.F. & Markram, H. (2005) Neuropeptide and calcium-binding protein gene expression profiles predict neuronal anatomical type in the juvenile rat. *J. Physiol.*, 567, 401-413.

Verdoorn, T.A. (1994) Formation of heteromeric γ-aminobutyric acid type A receptors containing two different α subunits. *Mol. Pharmacol.*, 45, 475-480.

Vicini, S., Ferguson, C., Prybylowski, K., Kralic, J.E., Morrow, A.L. & Homanics, G.E. (2001) $GABA_A$ receptor α1 subunit deletion prevents developmental changes of inhibitory synaptic currents in cerebellar neurons. *J. Neurosci.*, 21, 3009-3016.

Wisden, W., Laurie, D.J., Monyer, H. & Seeburg, P.H. (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neurosci.*, 12, 1040-1062.

General Discussion

In this thesis, the expression and functional role of different benzodiazepine-sensitive $GABA_A$ receptors in the CA1 area of the hippocampus has been investigated, as well as the changes that occur in $GABA_A$ receptor expression and function in interneurons to compensate for the complete loss of the α1-$GABA_A$ receptors.

We assessed the functional involvement of α1-, α2-, α3- and α5-$GABA_A$ receptors in phasic and tonic forms of inhibition in pyramidal cells and interneurons, using as strategy knock-in mice carrying specific $GABA_A$ receptor subtypes made insensitive to diazepam (Wieland et al., 1992; Benson et al., 1998; Rudolph et al., 1999). A change in diazepam effect compared with wildtype mice was taken as evidence for the presence of the corresponding $GABA_A$ receptor subtype in the investigated cell types. Our work shows that is possible to identify $GABA_A$ receptor subtypes that mediate phasic and tonic inhibitory neurotransmission in a defined cell type, and that the function of the receptor is closely correlated with its subcellular distribution (Brunig et al., 2002). These results also contribute to a better understanding of the pharmacological actions of diazepam in the hippocampus, in particular through the modulation of tonic inhibition.

The changes that occur in hippocampal interneurons to compensate for the complete loss of α1-$GABA_A$ receptors were investigated in knock-out mice with a targeted deletion of the α1 subunit (Vicini et al., 2001). We were able to show that the plasticity of $GABA_A$ receptors enabled them to preserve inhibitory transmission, and that this key component of the compensatory response is sufficient to protect against a pathological challenge such as kainic acid-induced seizures.

Altogether our results contribute to a better understanding of the organization and function of the $GABA_A$ergic system, a necessary requirement for the understanding of the neuronal basis of behaviour.

In the following, I will discuss the main findings of the different publications and their functional implications.

1. Functional role of $GABA_A$ receptors in pyramidal cells

We investigated whether the differential subcellular distribution of $GABA_A$ receptors in pyramidal cells implies a differential participation in phasic and tonic inhibition. Traditionally, inhibitory transmission refers to fast phasic inhibition, which results from the activation of receptors immediately beneath the release site. However, spillover (diffusion of the neurotransmitter out of the synaptic cleft) can also activate perisynaptic or extrasynaptic receptors, resulting in IPSCs with slower kinetics. Moreover, tonic inhibition resulting from a steady state of open receptors with high affinity for ambient GABA, and preferentially located at extrasynaptic sites, carries a major fraction of the total inhibitory currents. This mode of $GABA_A$ receptor activation indicates that inhibition is a complex functional process that can also take place in a less spatially and temporally restricted manner. In addition, the relative contribution of phasic and tonic inhibition varies in different regions of the brain and in different cell types, which adds extra complexity to the system. We found that α1-$GABA_A$ receptors and α2-$GABA_A$ receptors, which are synaptically located in hippocampal pyramidal cells, participate exclusively in fast phasic inhibition and their different subcellular locations enables them to receive inputs from segregated circuits. Thus α2-$GABA_A$ receptors, which are located predominantly on the axon initial segment of pyramidal cells and on soma (Brunig et al., 2002), are mostly activated by external stimulation proximal to the cell soma, whereas α1-$GABA_A$ receptors, which predominate in dendritic layers (Brunig et al., 2002), are mostly activated by external stimulation distal to the soma. The activation of α1-$GABA_A$ receptors and α2-$GABA_A$ receptors by specific interneurons shows that distinct $GABA_A$ receptor subtypes are involved in dendritic and somatic forms of inhibition, implying that they play a different role in synaptic integration and therefore modulate specific behavioural states. For example, it has been shown that somatic inhibition enforces precise coincidence detection of excitatory inputs to the soma, whereas dendritic inhibition sums incoming activity over broader time windows (Pouille and Scanziani, 2001). Also, recurrent inhibition can rapidly shift from soma to dendrites, recruiting two distinct inhibitory circuits according to the onset and rate of action potentials (Pouille and Scanziani, 2004). Additionally, *in vivo* studies have demonstrated that networks of different interneurons innervating distinct domains of pyramidal cells coordinate the activity of the cells (Klausberger et al., 2003) and are implicated in synchronizing cortical neurons at various frequencies through

different $GABA_A$ receptors (Somogyi and Klausberger, 2005). α3-$GABA_A$ receptors were immunohistochemically and functionally not detected in pyramidal cells.

We also showed that under intact GABA uptake, α5-$GABA_A$ receptors that are largely extrasynaptically located in pyramidal cells (Brunig et al., 2002; Crestani et al., 2002) participate in slow events and in tonic inhibition. Although a pool of α5-$GABA_A$ receptors has been proven to have a synaptic localization (Serwanski et al., 2006) and to mediate fast phasic inhibition from cells innervating distal pyramidal cells (Thomson et al., 2000), we could not detect a contribution of these receptors to fast phasic inhibition, probably because they mediate a minor fraction of sIPSCs; moreover the preferentially perisynaptic and extrasynaptic location was confirmed because under enhanced GABA spillover, more α5-$GABA_A$ receptors were recruited, and enhanced slow, but not fast, currents in the CA1 area.

We showed that slow events and tonic inhibition share the same pool of extrasynaptic receptors, because a low dose of picrotoxin preferentially blocked both slow events and tonic currents, but not fast events. This reflects the higher affinity of extrasynaptic receptors for GABA, and their persistent activation. Differences in tonic conductance recorded in CA1 adult hippocampal pyramidal cells are evident across species; under intact GABA uptake, pyramidal cells from guinea-pig do not show any tonic conductance (Semyanov et al., 2004), whereas in rats (Scimemi et al., 2005), and even more pronouncedly in mice (Stell and Mody, 2002; Caraiscos et al., 2004), tonic inhibition is detected.

The literature is not consistent on the effectiveness of different antagonists in selectively blocking phasic or tonic receptors in CA1 pyramidal cells. For example, gabazine (SR95531) is reported to selectively block phasic events at a concentration of 10 μM (Bai et al., 2001), but in our recordings, 10 μM gabazine completely blocked phasic and tonic conductances, which is in agreement with results from Stell and Mody (2002). Thus, to selectively block tonic or phasic inhibition, one needs to have into account not only the affinity of the receptors for the antagonist, but also the variability in ambient GABA according to the species, brain area and experimental conditions.

Slow sIPSCs occur infrequently and are difficult to evoke, suggesting that cells underlying these events have a low spontaneous firing rate and a unique position. The spatial restrictions of the axonal projections and the level of spontaneous firing activity of

different classes of interneurons provide the identity of the cells underlying fast and slow events. Basket cells, axo-axonic, bistratified and horizontal trilaminar cells underlie fast sIPSCs, because they project exclusively to somatic and perisomatic regions in the stratum pyramidale and in the proximal stratum radiatum. Interneurons in stratum lacunosum-moleculare are likely to underlie slow sIPSCs, because they project exclusively to dendritic regions. A possible candidate to generate slow IPSCs are the neurogliaform cells, which in the somatosensory cortex predominantly target the dendritic spines of pyramidal neurons and are capable of eliciting slow IPSPs through the combined recruitment of $GABA_A$ and $GABA_B$ receptors (Tamas et al., 2003).

The variability in kinetics in the sIPSCs may result from binding or gating properties of the $GABA_A$ receptors, but may also originate from fluctuations in the postsynaptic receptor number and/or in the transmitter concentration. Dendritic IPSCs are slower to decay because the transmitter is present in the synaptic cleft for an extended period of time. This hypothesis is consistent with our observation that the GABA uptake inhibitor NO711 prolonged the slow decay phase of slow sIPSCs.

The preferential extrasynaptic location of $\alpha5$-$GABA_A$ receptors, and therefore their role in tonic inhibition (Caraiscos et al., 2004; Prenosil et al., 2006; Marchionni et al., 2007), appears to be a critical element in the regulation of the acquisition and expression of associative memory. $\alpha5$ subunit knock-in mice, $\alpha5$(H105R), which have a partial deficit of $\alpha5$-$GABA_A$ receptors in the hippocampus, improved their performance in trace-fear conditioning, a hippocampus-dependent memory task (Crestani et al., 2002). In addition, these mutants display a resistance to the extinction of conditional fear over several days (Yee et al., 2004). Moreover, in $\alpha5$ knock-out mice, a significantly improved performance in a water-maze model of spatial learning has been observed (Collinson et al., 2002). Furthermore, compounds selective for $\alpha5$-$GABA_A$ receptors (L-655,708) have been reported to enhance the performance of wildtype rats in terms of encoding and recall processes in the Morris water-maze test (Chambers et al., 2004; Atack et al., 2006). In a mouse hippocampal slice model, L-655,708 was able to enhance the long-term potentiation produced by a theta burst stimulation, consistent with a potential role for the $\alpha5$-$GABA_A$ receptors in processes involving synaptic plasticity, such as learning and memory (Atack et al., 2006).

α5-$GABA_A$ receptors are also involved in: prepulse inhibition and locomotor activity (Hauser et al., 2005); tolerance to the sedative action of diazepam (van Rijnsoever et al., 2004); sedation (partially) (Savic et al., 2007); generation of γ oscillations (Towers et al., 2004) and epilepsy (Scimemi et al., 2005).

In most brain areas, tonic inhibition is modulated by δ-$GABA_A$ receptors (Brickley et al., 2001; Stell et al., 2003), which are diazepam-insensitive receptors potentiated by neurosteroids, with high affinity for GABA, slow desensitization and high sensitivity to ethanol (Wallner et al., 2003; Yeung et al., 2003; Mtchedlishvili and Kapur, 2006). However, in hippocampal pyramidal cells, α5-$GABA_A$ receptors, which are diazepam sensitive and have a lower affinity for GABA, were shown to be involved in tonic inhibition (Caraiscos et al., 2004; Prenosil et al., 2006). It should be noted, however, that only under increased GABA concentrations are α5-$GABA_A$ receptors involved in tonic inhibition in pyramidal cells (Caraiscos et al., 2004); at low ambient GABA concentrations, the activation of δ-subunit-containing $GABA_A$ receptors predominates (Scimemi et al., 2005). The relative contribution of diazepam-sensitive and diazepam-insensitive receptors to tonic inhibition in the same cell type implies an adaptive plasticity of the tonic current in the presence of enhanced or low ambient GABA, probably to ensure tonic current flow into pyramidal cells and avoid hyperexcitability. This is evidenced in mice lacking the α5 subunit, where a homeostatic upregulation of δ subunits maintains nearly 50% of residual tonic currents in CA1 pyramidal cells, preventing them from expressing hyperexcitability (Glykys and Mody, 2006). Our main findings of the study are summarized in Figure 1.

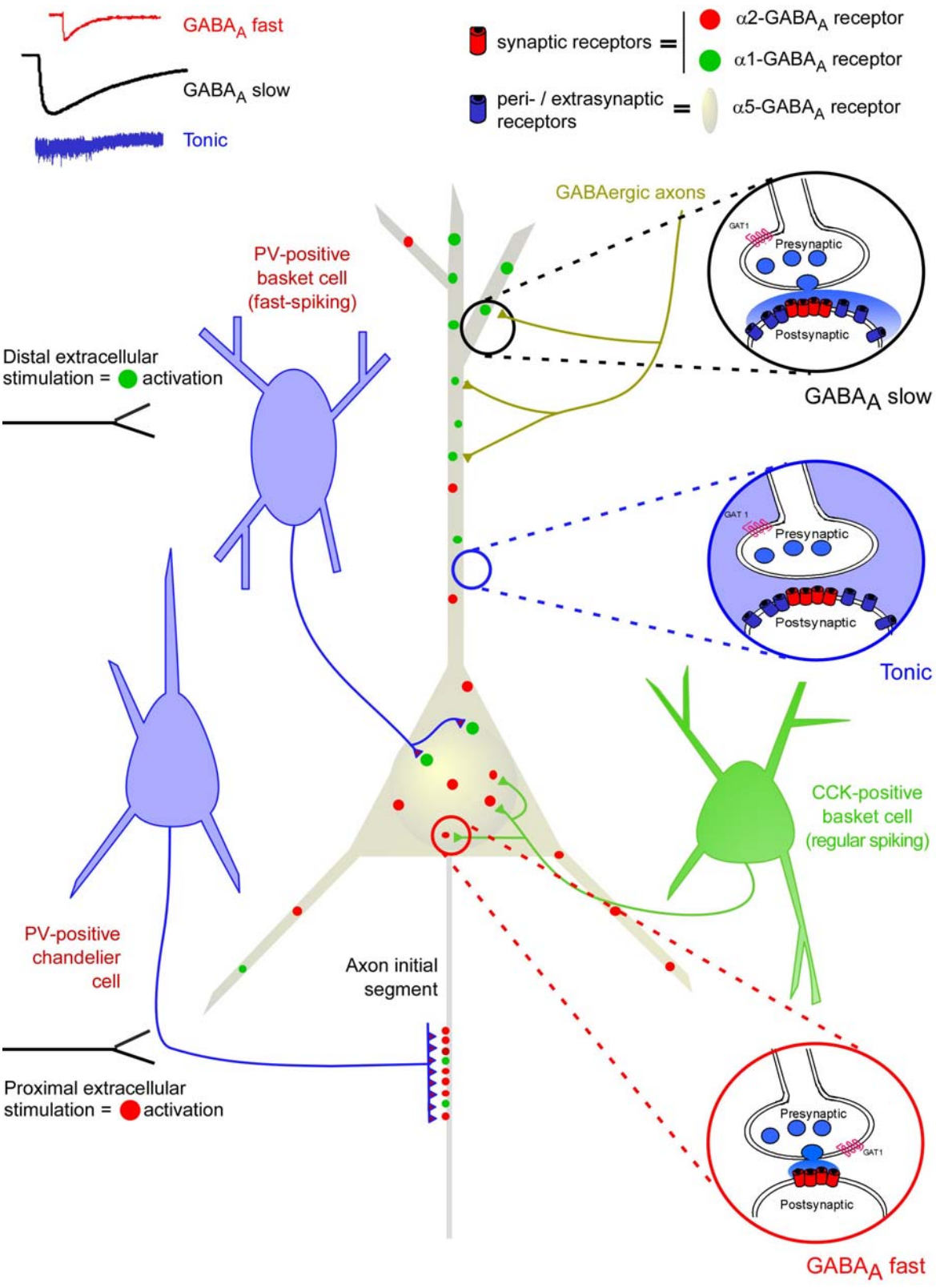


Figure 1. Diagrammatic representation of the subcellular distribution of $GABA_A$ receptor subtypes in CA1 hippocampal pyramidal cell and their established functional roles. CCK, cholecystokinin; PV, parvalbumin. Graph modified from (Fritschy and Brunig, 2003; Farrant and Nusser, 2005; Mohler et al., 2005; Rudolph and Mohler, 2006)

2. Tonic inhibition in interneurons

2.1. $GABA_A$ receptors involved in tonic inhibition in CA1 interneurons

We investigated the involvement of different $GABA_A$ receptor subtypes in tonic inhibition in interneurons. Morphological data shows that α1 and δ subunits are commonly expressed in interneurons; the α3 subunit is expressed in a subset of interneurons located in stratum oriens and hilus (Brunig et al., 2002); and the α2 and α5 subunits are virtually absent (Fritschy and Mohler, 1995; Pirker et al., 2000). We found that under intact GABA uptake most interneurons in stratum oriens and radiatum present tonic inhibition mediated by α1-$GABA_A$ receptors, confirming morphological data (Gao and Fritschy, 1994). According to the literature, two main types of α1-$GABA_A$ receptor assemblies could be present in interneurons: α1/δ assemblies for extrasynaptic diazepam-insensitive $GABA_A$ receptors involved in tonic inhibition (Glykys et al., 2007), and α1/γ2 for diazepam-sensitive receptors involved in phasic inhibition (Somogyi et al., 1996). We also showed that tonic inhibition was diazepam sensitive, so the subunit composition of extrasynaptic α1-$GABA_A$ receptors in CA1 interneurons includes $\alpha_1\beta_x\gamma_2$ in addition to $\alpha_1\beta_x\delta$–receptors. Moreover, although $\alpha_1\beta_x\gamma_2$ $GABA_A$ receptors are highly enriched in synapses, more receptors are found outside than inside synaptic junctions (Nusser et al., 1995). Triple immunostaining for the α1, δ and γ_2 subunits could clarify whether one or more α1-$GABA_A$ receptor assemblies are present in any given CA1 interneuron or whether they are segregated between different cell types. In double knock-in mice for the α2 and α3 subunits, most interneurons had an increase in holding current after diazepam application similar to wildtype mice, but in nearly 25% of the interneurons no effect of diazepam was observed. In double knock-in mice for the α1 and α2 subunits, the effect of diazepam on holding current was completely abolished in all except one of the interneurons. Thus α2-$GABA_A$ receptors may be involved in tonic inhibition in nearly 25% of the hippocampal interneurons. α2 subunits were reported to be mostly located in synaptic sites (Brunig et al., 2002) and to be absent in CA1 interneurons (Fritschy and Mohler, 1995), so the involvement of α2-$GABA_A$ receptors in tonic inhibition is a novel result. The α3 subunit could also be involved in tonic inhibition in the cells that were shown to express this subunit.

Altogether, our results showed: 1) α1-$GABA_A$ receptors, which are the most abundant in the brain, can also be involved in different functional roles according to their cellular and subcellular location. In pyramidal cells, α1-$GABA_A$ receptors are involved in phasic inhibition while in interneurons, they are mostly involved in tonic inhibition. 2) α2-$GABA_A$ receptors are involved in phasic inhibition in pyramidal cells and are present in dendrites from several interneurons clustered and colocalized with gephyrin, thus they are assumed to be synaptically located. In some interneurons, α2-$GABA_A$ receptors also participate in tonic inhibition. 3) α3-$GABA_A$ receptors are absent in pyramidal cells and most interneurons. 4) α5-$GABA_A$ receptors are only present in pyramidal cells and are involved in slow inhibitory currents and in tonic inhibition. α5-$GABA_A$ receptors are mostly activated under conditions of raised extracellular GABA. The apparent involvement of α1- and α2-$GABA_A$ receptors containing the γ2 subunit in tonic inhibition opens new questions about the functional role of $GABA_A$ receptors. If several $GABA_A$ receptor isoforms are indeed responsible for tonic currents, this could be an attractive target for the development of improved drug therapies against pathologies such as epilepsy.

2.2. Strata differences in tonic conductance in interneurons: Functional role of GABA transporters?

In the second study we also showed that under intact GABA uptake, interneurons in stratum oriens and stratum radiatum differ in their extent of tonic conductance. Interneurons in stratum oriens showed nearly double the amount of tonic conductance compared to interneurons in stratum radiatum. In stratum lacunosum-moleculare, some cells completely lacked tonic conductance. As indicated previously, α1-$GABA_A$ receptors were mostly involved in tonic inhibition in interneurons, so the layer-specific difference in tonic currents was not due to differences in $GABA_A$ receptor composition (desensitization and mean open time). Rather, it resulted from differences in GABA release or uptake. It has been shown that changes in presynaptic activity and release modify the magnitude of the tonic conductance. For example, an increase in interneuron firing also increases tonic conductance in pyramidal cells (Kullmann and Semyanov, 2002), whereas the blockade of action-potential firing reduces tonic conductance (Petrini et al., 2004). Likewise in cerebellar granule cells, the facilitation of GABA release causes an increase in tonic conductance (Rossi et al., 2003). Inhibitory interneurons can fire repetitively at rates much

higher than excitatory neurons and with a higher probability of release. This means that interneurons with a high-frequency train of action potentials should have longer-lasting transmitter release and thereby more GABA could spill out of the synaptic cleft and contribute to tonic inhibition. To confirm this hypothesis, I tried to investigate the different firing patterns of interneurons present in each stratum. In stratum oriens, most registered interneurons did indeed have a spiking-pattern frequency between 40 and 60 Hz, whereas in stratum radiatum, most interneurons had a spiking-pattern frequency between 10 and 20 Hz.

The firing properties of a cell do not dictate its tonic inhibitory conductance; interneurons in stratum radiatum and lacunosum-moleculare showed a consistently reduced tonic conductance and larger cell capacitance independent of their spiking properties, but a group of high-frequency firing interneurons located in stratum lacunosum-moleculare lacked tonic conductance. This indicates that tonic conductance can be cell-type specific.
Another source of variability between strata are the local GABAergic inputs. It is possible that stratum oriens interneurons receive more inputs than stratum radiatum and lacunosum-moleculare interneurons, reflected by the higher amount of interneurons present in stratum oriens.

A second possible explanation for the strata difference in tonic inhibition lies in the uptake by GABA transporters. There has been relatively little focus on the role of GABA transporters in regulating the level of tonic inhibition in the hippocampus. In rat CA1 area, a lower expression of GAT-1 mRNA (the main neuronal GABA plasma-membrane transporter) in interneurons of stratum oriens compared with those of stratum radiatum/lacunosum-moleculare was observed (Engel et al., 1998). It has also been shown that GABA transporters modulate the level of tonic inhibition by trying to maintain ambient GABA concentrations close to a set point, such as the resting potential of the cells (Richerson and Wu, 2003). This means that the equilibrium for the extracellular GABA concentration changes to modulate the level of tonic inhibition in response to neuronal activity and in response to the transmembrane gradients for GABA, chloride and sodium. It is likely that the function of GABA transporters varies among different types of interneurons as it varies among different types of synapses (Chiu et al., 2002). To better support the possibility of changes in GAT-1 expression according to strata and/or according to interneuron type, *in situ* hybridization with mRNA specific for GAT-1 in GAD67-GFP mice would be necessary. Another complementary approach would be to

measure the effect of NO-711 on IPSCs from stratum oriens and stratum radiatum interneurons.

The dynamic properties of GABA transporters are an extra complexity in the inhibitory system and are a potential source of investigation, because these molecules contribute to a variety of pathological conditions and are the targets of several drugs. In epilepsy, for example, reversal of the GABA transporter is likely to occur during seizures and regulation of tonic inhibition may modulate seizure threshold. To know whether anticonvulsant drugs such as tiagabine also block the transporter reversal is of greatest significance in this field. Tonic inhibition is also highly sensitive to modulation by other clinically relevant compounds, like endogenous neuroactive steroids (Stell et al., 2003), intravenous and inhalation anaesthetics, and ethanol (Sundstrom-Poromaa et al., 2002; Glykys et al., 2007). So far most δ-$GABA_A$ receptors have been shown to be selectively modulated by neurosteroids and ethanol, but tonic activation can probably also involve other types of receptors depending on the level of ambient GABA.

3. Plasticity of the GABAergic system

The results from the functional studies are in line with previous results showing that $GABA_A$ receptor subtypes are tailor-made to fulfil specific functional requirements. In the third part of the thesis, I therefore investigated how the GABAergic system copes with the deletion of the α1 subunit gene. To this end I developed a new immunostaining protocol based on the weak fixation of acute brain slices (Publication 3). With this new protocol, an optimal simultaneous detection of neurochemical markers (PV, CR, CB, NPY and SOM), $GABA_A$ receptors and gephyrin was achieved in triple-labelling experiments. The preparation of living slices prior to weak tissue fixation, helped to preserve the cell morphology while minimising epitope masking of postsynaptic proteins, which occurs in perfusion-fixed tissue.

In all identified interneurons in wildtype mice, α3, α4 and α5 subunits were absent but α2 subunits were clustered and colocalized with gephyrin predominantly on the dendrites of several interneurons. Combined with the electrophysiological results, this finding extends our knowledge of the expression of $GABA_A$ receptors by demonstrating the existence of α2-$GABA_A$ receptors in interneurons.

Although the α1 subunit is the most abundant (around 50%), $\alpha1^{0/0}$ mice exhibit a mild phenotype due in part to compensatory alterations in the GABAergic system (Kralic et al., 2002; Kralic et al., 2006; Ogris et al., 2006). In $\alpha1^{0/0}$ mice a general compensatory increase in α2, α3 and α4 subunits is observed in different brain areas with unchanged regional and subcellular distributions (Kralic et al., 2006). In $\alpha1^{0/0}$ mice, a replacement of the α1 by the α3 subunit occurred in interneurons that normally express the α1 subunit: 100% PV-, 53 ± 5 % CR-, 33 ± 8 % CB-; 57 ± 4 % NPY- and 64 ± 6 % SOM-positive cells. Hippocampal interneurons normally do not show α3 subunit expression. However it is unlikely that the upregulation of the α3 subunit resulted from a change in gene expression, most probably all interneurons in the hippocampus do express the α3 subunit at very low levels that are undetectable by immunostaining techniques. In order to know at which level the compensatory changes occurred in hippocampal interneurons, further studies from gene and mRNA expression should be performed.

All up-regulated α3-$GABA_A$ receptors in interneurons were clustered and colocalized with gephyrin, suggesting that an increase in synaptic inputs had occurred, perhaps by a switch of tonic towards phasic signalling. Surprisingly, sIPSC recordings showed no change either in tonic signalling or in the number of phasic events compared to wildtype mice. α2- and α3-$GABA_A$ receptors have a lower affinity for GABA than α1-$GABA_A$ receptors (Ebert et al., 1994; Verdoorn, 1994), implying that their level of synaptic activation could be low, and therefore more synaptic inputs were required to compensate for the loss of α1-$GABA_A$ receptors. Moreover the slower kinetics of α3-$GABA_A$ receptors and their slow desensitization may be enough to preserve the tonic conductance. This receptor reorganization suggests a rearrangement of GABAergic circuits. Interestingly, when kainic acid was applied to induce epilepsy, no difference in severity or duration of status epilepticus was observed in $\alpha1^{0/0}$ compared to wildtype mice, confirming that the $GABA_A$ mediated transmission was well preserved.

This study also showed an increase in α2 synaptic receptors on interneurons that are normally α2 positive, and on pyramidal cells. Therefore an overall increase of synaptic inputs is seen in $\alpha1^{0/0}$ mice in both pyramidal cells and interneurons. The increase in synaptic inputs is cell specific, since the density of gephyrin clusters in CB- and SOM-positive interneurons remains unchanged. Apparently, in $\alpha1^{0/0}$ mice, the α2 subunit is overexpressed on α2 positive cells and the α3 subunit is upregulated on normally α1-

positive cells. In the $\alpha1^{0/0}$ mice, mIPSCs showed a reduced amplitude and frequency of events compared to wildtype, suggesting either that many events fell into the noise level due to the changes in biophysical properties of the α2- and α3-$GABA_A$ receptors or that there was impaired GABAergic signalling compared to wildtype mice. The reduced frequency is not observed under normal action-potential release because the high GABA levels can saturate all synaptic receptors. The reduced amplitude and frequency could also be attributed to a change in quantal size so that in $\alpha1^{0/0}$ mice, a reduction in GABA vesicular contents may occur. No direct proof of this hypothesis exists, but it cannot be excluded that presynaptic changes occur in response to a loss of postsynaptic $GABA_A$ receptors. Future investigations of GABA quantal size and content, and the influence of postsynaptic $GABA_A$ receptor expression, will consolidate our understanding of the homeostatic inhibitory machinery.

Altogether, our results showed the remarkable plasticity of the $GABA_A$ergic system in the hippocampus to maintain a normal phenotype in the absence of a major $GABA_A$ receptor subtype. This plasticity includes not only expression of other $GABA_A$ receptors but also an increase in synaptic connections and therefore a total rearrangement of the neuronal networks. This network rearrangement seems to be sufficient to prevent overexcitation, as observed by the similar response to kainic acid injection in wildtype and $\alpha1^{0/0}$ mice but is well probable that other adaptatory changes like in different channel densities may also occur in $\alpha1^{0/0}$ hippocampal interneurons.

References

Atack JR, Bayley PJ, Seabrook GR, Wafford KA, McKernan RM, Dawson GR (2006) L-655,708 enhances cognition in rats but is not proconvulsant at a dose selective for α5-containing $GABA_A$ receptors. Neuropharmacology 51:1023-1029.

Auger C, Kondo S, Marty A (1998) Multivesicular release at single functional synaptic sites in cerebellar stellate and basket cells. J Neurosci 18:4532-4547.

Bai D, Zhu G, Pennefather P, Jackson MF, MacDonald JF, Orser BA (2001) Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by $GABA_A$ receptors in hippocampal neurons. Mol Pharmacol 59:814-824.

Banks MI, Pearce RA (2000) Kinetic differences between synaptic and extrasynaptic $GABA_A$ receptors in CA1 pyramidal cells. J Neurosci 20:937-948.

Barberis A, Lu C, Vicini S, Mozrzymas JW (2005) Developmental changes of GABA synaptic transient in cerebellar granule cells. Mol Pharmacol 67:1221-1228.

Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN, Langer SZ (1998) International Union of Pharmacology. XV. Subtypes of $GABA_A$ receptors: classification on the basis of subunit structure and receptor function. Pharmacol Rev 50:291-313.

Belelli D, Casula A, Ling A, Lambert JJ (2002) The influence of subunit composition on the interaction of neurosteroids with $GABA_A$ receptors. Neuropharmacology 43:651-661.

Ben-Ari Y (2002) Excitatory actions of GABA during development: the nature of the nurture. Nat Rev Neurosci 3:728-739.

Benke D, Fritschy JM, Trzeciak A, Bannwarth W, Mohler H (1994) Distribution, prevalence, and drug binding profile of $GABA_A$ receptor subtypes differing in the β-subunit variant. J Biol Chem 269:27100-27107.

Benson JA, Low K, Keist R, Mohler H, Rudolph U (1998) Pharmacology of recombinant $GABA_A$ receptors rendered diazepam-insensitive by point-mutated α-subunits. FEBS Lett 431:400-404.

Bonnert TP, McKernan RM, Farrar S, le Bourdelles B, Heavens RP, Smith DW, Hewson L, Rigby MR, Sirinathsinghji DJ, Brown N, Wafford KA, Whiting PJ (1999) τ, a novel $GABA_A$ receptor subunit. Proc Natl Acad Sci U S A 96:9891-9896.

Bormann J (1986) Electrophysiology of single GABA receptor chloride channels. Clin Neuropharmacol 9 Suppl 4:386-388.

Bormann J (2000) The 'ABC' of GABA receptors. Trends Pharmacol Sci 21:16-19.

Bosman LW, Heinen K, Spijker S, Brussaard AB (2005) Mice lacking the major adult $GABA_A$ receptor subtype have normal number of synapses, but retain juvenile IPSC kinetics until adulthood. J Neurophysiol 94:338-346.

Bouilleret V, Ridoux V, Depaulis A, Marescaux C, Nehlig A, Le Gal La Salle G (1999) Recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult mice: electroencephalography, histopathology and synaptic reorganization similar to mesial temporal lobe epilepsy. Neuroscience 89:717-729.

Brickley SG, Cull-Candy SG, Farrant M (1996) Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of $GABA_A$ receptors. J Physiol 497 (Pt 3):753-759.

Brickley SG, Revilla V, Cull-Candy SG, Wisden W, Farrant M (2001) Adaptive regulation of neuronal excitability by a voltage-independent potassium conductance. Nature 409:88-92.

Brown N, Kerby J, Bonnert TP, Whiting PJ, Wafford KA (2002) Pharmacological characterization of a novel cell line expressing human $\alpha4\beta3\delta$ $GABA_A$ receptors. Br J Pharmacol 136:965-974.

Brunig I, Scotti E, Sidler C, Fritschy JM (2002) Intact sorting, targeting, and clustering of $GABA_A$ receptor subtypes in hippocampal neurons *in vitro*. J Comp Neurol 443:43-55.

Buzsaki G (1989) Two-stage model of memory trace formation: a role for "noisy" brain states. Neuroscience 31:551-570.

Buzsaki G (2002) Theta oscillations in the hippocampus. Neuron 33:325-340.

Buzsaki G, Chrobak JJ (1995) Temporal structure in spatially organized neuronal ensembles: a role for interneuronal networks. Curr Opin Neurobiol 5:504-510.

Buzsaki G, Leung LW, Vanderwolf CH (1983) Cellular bases of hippocampal EEG in the behaving rat. Brain Res 287:139-171.

Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF, Orser BA (2004) Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by $\alpha5$ subunit-containing $GABA_A$ receptors. Proc Natl Acad Sci U S A 101:3662-3667.

Chambers MS, Atack JR, Carling RW, Collinson N, Cook SM, Dawson GR, Ferris P, Hobbs SC, O'Connor D, Marshall G, Rycroft W, Macleod AM (2004) An orally bioavailable, functionally selective inverse agonist at the benzodiazepine site of $GABA_A$ α5 receptors with cognition enhancing properties. J Med Chem 47:5829-5832.

Chandra D, Jia F, Liang J, Peng Z, Suryanarayanan A, Werner DF, Spigelman I, Houser CR, Olsen RW, Harrison NL, Homanics GE (2006b) $GABA_A$ receptor α4 subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. Proc Natl Acad Sci U S A 103:15230-15235.

Chavas J, Marty A (2003) Coexistence of excitatory and inhibitory GABA synapses in the cerebellar interneuron network. J Neurosci 23:2019-2031.

Chebib M, Johnston GA (1999) The 'ABC' of GABA receptors: a brief review. Clin Exp Pharmacol Physiol 26:937-940.

Chiu CS, Jensen K, Sokolova I, Wang D, Li M, Deshpande P, Davidson N, Mody I, Quick MW, Quake SR, Lester HA (2002) Number, density, and surface/cytoplasmic distribution of GABA transporters at presynaptic structures of knock-in mice carrying GABA transporter subtype 1-green fluorescent protein fusions. J Neurosci 22:10251-10266.

Collinson N, Kuenzi FM, Jarolimek W, Maubach KA, Cothliff R, Sur C, Smith A, Otu FM, Howell O, Atack JR, McKernan RM, Seabrook GR, Dawson GR, Whiting PJ, Rosahl TW (2002) Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the α5 subunit of the $GABA_A$ receptor. J Neurosci 22:5572-5580.

Crestani F, Keist R, Fritschy JM, Benke D, Vogt K, Prut L, Bluthmann H, Mohler H, Rudolph U (2002) Trace fear conditioning involves hippocampal α5-$GABA_A$ receptors. Proc Natl Acad Sci U S A 99:8980-8985.

Crestani F, Low K, Keist R, Mandelli M, Mohler H, Rudolph U (2001) Molecular targets for the myorelaxant action of diazepam. Mol Pharmacol 59:442-445.

Crestani F, Martin JR, Mohler H, Rudolph U (2000) Mechanism of action of the hypnotic zolpidem *in vivo*. Br J Pharmacol 131:1251-1254.

Csicsvari J, Hirase H, Czurko A, Mamiya A, Buzsaki G (1999) Oscillatory coupling of hippocampal pyramidal cells and interneurons in the behaving Rat. J Neurosci 19:274-287.

Csicsvari J, Jamieson B, Wise KD, Buzsaki G (2003) Mechanisms of γ oscillations in the hippocampus of the behaving rat. Neuron 37:311-322.

Demarque M, Represa A, Becq H, Khalilov I, Ben-Ari Y, Aniksztejn L (2002) Paracrine intercellular communication by a Ca2+- and SNARE-independent release of GABA and glutamate prior to synapse formation. Neuron 36:1051-1061.

Devor A, Fritschy JM, Yarom Y (2001) Spatial distribution and subunit composition of $GABA_A$ receptors in the inferior olivary nucleus. J Neurophysiol 85:1686-1696.

Diana MA, Marty A (2004) Endocannabinoid-mediated short-term synaptic plasticity: depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE). Br J Pharmacol 142:9-19.

Ebert B, Wafford KA, Whiting PJ, Krogsgaard-Larsen P, Kemp JA (1994) Molecular pharmacology of $GABA_A$ receptor agonists and partial agonists in oocytes injected with different α, β, and γ receptor subunit combinations. Mol Pharmacol 46:957-963.

Eichenbaum H (2000) Hippocampus: mapping or memory? Curr Biol 10:R785-787.

Engel D, Schmitz D, Gloveli T, Frahm C, Heinemann U, Draguhn A (1998) Laminar difference in GABA uptake and GAT-1 expression in rat CA1. J Physiol 512 (Pt 3):643-649.

Essrich C, Lorez M, Benson JA, Fritschy JM, Luscher B (1998) Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the γ2 subunit and gephyrin. Nat Neurosci 1:563-571.

Farrant M, Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. Nat Rev Neurosci 6:215-229.

Farrar SJ, Whiting PJ, Bonnert TP, McKernan RM (1999) Stoichiometry of a ligand-gated ion channel determined by fluorescence energy transfer. J Biol Chem 274:10100-10104.

Freund TF (2003) Interneuron Diversity series: Rhythm and mood in perisomatic inhibition. Trends Neurosci 26:489-495.

Freund TF, Buzsaki G (1996) Interneurons of the hippocampus. Hippocampus 6:347-470.

Fritschy JM, Brunig I (2003) Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications. Pharmacol Ther 98:299-323.

Fritschy JM, Johnson DK, Mohler H, Rudolph U (1998) Independent assembly and subcellular targeting of $GABA_A$-receptor subtypes demonstrated in mouse hippocampal and olfactory neurons *in vivo*. Neurosci Lett 249:99-102.

Fritschy JM, Mohler H (1995) $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. J Comp Neurol 359:154-194.

Fritschy JM, Panzanelli P, Kralic JE, Vogt KE, Sassoe-Pognetto M (2006) Differential dependence of axo-dendritic and axo-somatic GABAergic synapses on $GABA_A$ receptors containing the α1 subunit in Purkinje cells. J Neurosci 26:3245-3255.

Gao B, Fritschy JM (1994) Selective allocation of $GABA_A$ receptors containing the α1 subunit to neurochemically distinct subpopulations of rat hippocampal interneurons. Eur J Neurosci 6:837-853.

Glykys J, Mody I (2006) Hippocampal network hyperactivity after selective reduction of tonic inhibition in $GABA_A$ receptor α5 subunit-deficient mice. J Neurophysiol 95:2796-2807.

Glykys J, Peng Z, Chandra D, Homanics GE, Houser CR, Mody I (2007) A new naturally occurring $GABA_A$ receptor subunit partnership with high sensitivity to ethanol. Nat Neurosci 10:40-48.

Goldstein PA, Elsen FP, Ying SW, Ferguson C, Homanics GE, Harrison NL (2002) Prolongation of hippocampal miniature inhibitory postsynaptic currents in mice lacking the $GABA_A$ receptor α1 subunit. J Neurophysiol 88:3208-3217.

Gulledge AT, Stuart GJ (2003) Excitatory actions of GABA in the cortex. Neuron 37:299-309.

Gulyas AI, Megias M, Emri Z, Freund TF (1999) Total number and ratio of excitatory and inhibitory synapses converging onto single interneurons of different types in the CA1 area of the rat hippocampus. J Neurosci 19:10082-10097.

Hajos N, Nusser Z, Rancz EA, Freund TF, Mody I (2000) Cell type- and synapse-specific variability in synaptic $GABA_A$ receptor occupancy. Eur J Neurosci 12:810-818.

Hamann M, Rossi DJ, Attwell D (2002) Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex. Neuron 33:625-633.

Hanchar HJ, Dodson PD, Olsen RW, Otis TS, Wallner M (2005) Alcohol-induced motor impairment caused by increased extrasynaptic $GABA_A$ receptor activity. Nat Neurosci 8:339-345.

Hansra N, Arya S, Quick MW (2004) Intracellular domains of a rat brain GABA transporter that govern transport. J Neurosci 24:4082-4087.

Harris KD, Henze DA, Hirase H, Leinekugel X, Dragoi G, Czurko A, Buzsaki G (2002) Spike train dynamics predicts theta-related phase precession in hippocampal pyramidal cells. Nature 417:738-741.

Hauser J, Rudolph U, Keist R, Mohler H, Feldon J, Yee BK (2005) Hippocampal α5 subunit-containing $GABA_A$ receptors modulate the expression of prepulse inhibition. Mol Psychiatry 10:201-207.

Huopaniemi L, Keist R, Randolph A, Certa U, Rudolph U (2004) Diazepam-induced adaptive plasticity revealed by α1-$GABA_A$ receptor-specific expression profiling. J Neurochem 88:1059-1067.

Kaneda M, Farrant M, Cull-Candy SG (1995) Whole-cell and single-channel currents activated by GABA and glycine in granule cells of the rat cerebellum. J Physiol 485 (Pt 2):419-435.

Klausberger T, Magill PJ, Marton LF, Roberts JD, Cobden PM, Buzsaki G, Somogyi P (2003) Brain-state- and cell type-specific firing of hippocampal interneurons *in vivo*. Nature 421:844-848.

Klausberger T, Marton LF, Baude A, Roberts JD, Magill PJ, Somogyi P (2004) Spike timing of dendrite-targeting bistratified cells during hippocampal network oscillations *in vivo*. Nat Neurosci 7:41-47.

Klausberger T, Roberts JD, Somogyi P (2002) Cell type- and input-specific differences in the number and subtypes of synaptic $GABA_A$ receptors in the hippocampus. J Neurosci 22:2513-2521.

Kopp C, Rudolph U, Keist R, Tobler I (2003) Diazepam-induced changes on sleep and the EEG spectrum in mice: role of the α3-$GABA_A$ receptor subtype. Eur J Neurosci 17:2226-2230.

Kopp C, Rudolph U, Low K, Tobler I (2004b) Modulation of rhythmic brain activity by diazepam: $GABA_A$ receptor subtype and state specificity. Proc Natl Acad Sci U S A 101:3674-3679.

Kopp C, Rudolph U, Tobler I (2004a) Sleep EEG changes after zolpidem in mice. Neuroreport 15:2299-2302.

Korpi ER, Kuner T, Seeburg PH, Luddens H (1995) Selective antagonist for the cerebellar granule cell-specific $GABA_A$ receptor. Mol Pharmacol 47:283-289.

Kralic JE, Korpi ER, O'Buckley TK, Homanics GE, Morrow AL (2002a) Molecular and pharmacological characterization of $GABA_A$ receptor α1 subunit knock-out mice. J Pharmacol Exp Ther 302:1037-1045.

Kralic JE, O'Buckley TK, Khisti RT, Hodge CW, Homanics GE, Morrow AL (2002b) $GABA_A$ receptor α1 subunit deletion alters receptor subtype assembly, pharmacological and behavioral responses to benzodiazepines and zolpidem. Neuropharmacology 43:685-694.

Kralic JE, Sidler C, Parpan F, Homanics GE, Morrow AL, Fritschy JM (2006) Compensatory alteration of inhibitory synaptic circuits in cerebellum and thalamus of $GABA_A$ receptor α1 subunit knock-out mice. J Comp Neurol 495:408-421.

Kralic JE, Wheeler M, Renzi K, Ferguson C, O'Buckley TK, Grobin AC, Morrow AL, Homanics GE (2003) Deletion of $GABA_A$ receptor α1 subunit-containing receptors alters responses to ethanol and other anesthetics. J Pharmacol Exp Ther 305:600-607.

Kullmann DM, Semyanov A (2002) Glutamatergic modulation of GABAergic signaling among hippocampal interneurons: novel mechanisms regulating hippocampal excitability. Epilepsia 43 Suppl 5:174-178.

Li XG, Somogyi P, Ylinen A, Buzsaki G (1994) The hippocampal CA3 network: an *in vivo* intracellular labeling study. J Comp Neurol 339:181-208.

Loup F, Wieser HG, Yonekawa Y, Aguzzi A, Fritschy JM (2000) Selective alterations in $GABA_A$ receptor subtypes in human temporal lobe epilepsy. J Neurosci 20:5401-5419.

Lovinger DM (1997) Alcohols and neurotransmitter gated ion channels: past, present and future. Naunyn Schmiedebergs Arch Pharmacol 356:267-282.

Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, Fritschy JM, Rulicke T, Bluethmann H, Mohler H, Rudolph U (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. Science 290:131-134.

Macdonald RL, Barker JL (1978) Specific antagonism of GABA-mediated postsynaptic inhibition in cultured mammalian spinal cord neurons: a common mode of convulsant action. Neurology 28:325-330.

Marchionni I, Omrani A, Cherubini E (2007) In the developing rat hippocampus a tonic $GABA_A$-mediated conductance selectively enhances the glutamatergic drive of principal cells. J Physiol.

Markram H, Toledo-Rodriguez M, Wang Y, Gupta A, Silberberg G, Wu C (2004) Interneurons of the neocortical inhibitory system. Nat Rev Neurosci 5:793-807.

Marowsky A, Fritschy JM, Vogt KE (2004) Functional mapping of $GABA_A$ receptor subtypes in the amygdala. Eur J Neurosci 20:1281-1289.

Martina M, Vida I, Jonas P (2000) Distal initiation and active propagation of action potentials in interneuron dendrites. Science 287:295-300.

McBain CJ, Fisahn A (2001) Interneurons unbound. Nat Rev Neurosci 2:11-23.

Miles R, Toth K, Gulyas AI, Hajos N, Freund TF (1996) Differences between somatic and dendritic inhibition in the hippocampus. Neuron 16:815-823.

Mody I, Pearce RA (2004) Diversity of inhibitory neurotransmission through $GABA_A$ receptors. Trends Neurosci 27:569-575.

Mohler H (2006) $GABA_A$ receptor diversity and pharmacology. Cell Tissue Res 326:505-516.

Mohler H, Fritschy JM, Rudolph U (2002) A new benzodiazepine pharmacology. J Pharmacol Exp Ther 300:2-8.

Mohler H, Fritschy JM, Vogt K, Crestani F, Rudolph U (2005) Pathophysiology and pharmacology of $GABA_A$ receptors. Handb Exp Pharmacol:225-247.

Morin F, Beaulieu C, Lacaille JC (1996) Membrane properties and synaptic currents evoked in CA1 interneuron subtypes in rat hippocampal slices. J Neurophysiol 76:1-16.

Morris HV, Dawson GR, Reynolds DS, Atack JR, Stephens DN (2006) Both $\alpha 2$ and $\alpha 3$-$GABA_A$ receptor subtypes mediate the anxiolytic properties of benzodiazepine site ligands in the conditioned emotional response paradigm. Eur J Neurosci 23:2495-2504.

Mtchedlishvili Z, Kapur J (2006) High-affinity, slowly desensitizing $GABA_A$ receptors mediate tonic inhibition in hippocampal dentate granule cells. Mol Pharmacol 69:564-575.

Newland CF, Cull-Candy SG (1992) On the mechanism of action of picrotoxin on GABA receptor channels in dissociated sympathetic neurones of the rat. J Physiol 447:191-213.

Nusser Z, Cull-Candy S, Farrant M (1997) Differences in synaptic $GABA_A$ receptor number underlie variation in GABA mini amplitude. Neuron 19:697-709.

Nusser Z, Mody I (2002) Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. J Neurophysiol 87:2624-2628.

Nusser Z, Naylor D, Mody I (2001) Synapse-specific contribution of the variation of transmitter concentration to the decay of inhibitory postsynaptic currents. Biophys J 80:1251-1261.

Nusser Z, Roberts JD, Baude A, Richards JG, Somogyi P (1995) Relative densities of synaptic and extrasynaptic $GABA_A$ receptors on cerebellar granule cells as determined by a quantitative immunogold method. J Neurosci 15:2948-2960.

Nusser Z, Sieghart W, Somogyi P (1998) Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. J Neurosci 18:1693-1703.

Nyiri G, Freund TF, Somogyi P (2001) Input-dependent synaptic targeting of α2-subunit-containing $GABA_A$ receptors in synapses of hippocampal pyramidal cells of the rat. Eur J Neurosci 13:428-442.

Ogris W, Lehner R, Fuchs K, Furtmuller B, Hoger H, Homanics GE, Sieghart W (2006) Investigation of the abundance and subunit composition of $GABA_A$ receptor subtypes in the cerebellum of α1-subunit-deficient mice. J Neurochem 96:136-147.

Ortinski PI, Turner JR, Barberis A, Motamedi G, Yasuda RP, Wolfe BB, Kellar KJ, Vicini S (2006) Deletion of the $GABA_A$ receptor α1 subunit increases tonic $GABA_A$ receptor current: a role for GABA uptake transporters. J Neurosci 26:9323-9331.

Owens DF, Kriegstein AR (2002) Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3:715-727.

Owens DF, Liu X, Kriegstein AR (1999) Changing properties of $GABA_A$ receptor-mediated signaling during early neocortical development. J Neurophysiol 82:570-583.

Pangratz-Fuehrer S, Rudolph U, Huguenard JR (2007) Giant spontaneous depolarizing potentials in the developing thalamic reticular nucleus. J Neurophysiol 97:2364-2372.

Parra P, Gulyas AI, Miles R (1998) How many subtypes of inhibitory cells in the hippocampus? Neuron 20:983-993.

Pearce RA (1993) Physiological evidence for two distinct $GABA_A$ responses in rat hippocampus. Neuron 10:189-200.

Petrini EM, Marchionni I, Zacchi P, Sieghart W, Cherubini E (2004) Clustering of extrasynaptic $GABA_A$ receptors modulates tonic inhibition in cultured hippocampal neurons. J Biol Chem 279:45833-45843.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) $GABA_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101:815-850.

Porcello DM, Huntsman MM, Mihalek RM, Homanics GE, Huguenard JR (2003) Intact synaptic GABAergic inhibition and altered neurosteroid modulation of thalamic relay neurons in mice lacking δ subunit. J Neurophysiol 89:1378-1386.

Pouille F, Scanziani M (2001) Enforcement of temporal fidelity in pyramidal cells by somatic feed-forward inhibition. Science 293:1159-1163.

Pouille F, Scanziani M (2004) Routing of spike series by dynamic circuits in the hippocampus. Nature 429:717-723.

Prenosil GA, Schneider Gasser EM, Rudolph U, Keist R, Fritschy JM, Vogt KE (2006) Specific subtypes of $GABA_A$ receptors mediate phasic and tonic forms of inhibition in hippocampal pyramidal neurons. J Neurophysiol 96:846-857.

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, Seeburg PH (1989) Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. Nature 338:582-585.

Puia G, Vicini S, Seeburg PH, Costa E (1992) Different sites of action of neurosteroids and benzodiazepines on natural and recombinant $GABA_A$ receptors. Adv Biochem Psychopharmacol 47:103-110.

Quick MW, Hu J, Wang D, Zhang HY (2004) Regulation of a γ-aminobutyric acid transporter by reciprocal tyrosine and serine phosphorylation. J Biol Chem 279:15961-15967.

Richerson GB, Wu Y (2003) Dynamic equilibrium of neurotransmitter transporters: not just for reuptake anymore. J Neurophysiol 90:1363-1374.

Rivera C, Voipio J, Kaila K (2005) Two developmental switches in GABAergic signalling: the K+-Cl- cotransporter KCC2 and carbonic anhydrase CAVII. J Physiol 562:27-36.

Rogers CJ, Twyman RE, Macdonald RL (1994) Benzodiazepine and β-carboline regulation of single $GABA_A$ receptor channels of mouse spinal neurones in culture. J Physiol 475:69-82.

Rossi DJ, Hamann M, Attwell D (2003) Multiple modes of GABAergic inhibition of rat cerebellar granule cells. J Physiol 548:97-110.

Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Mohler H (1999) Benzodiazepine actions mediated by specific γ-aminobutyric acid(A) receptor subtypes. Nature 401:796-800.

Rudolph U, Mohler H (2006) GABA-based therapeutic approaches: $GABA_A$ receptor subtype functions. Curr Opin Pharmacol 6:18-23.

Savic MM, Huang S, Furtmuller R, Clayton T, Huck S, Obradovic DI, Ugresic ND, Sieghart W, Bokonjic DR, Cook JM (2007) Are $GABA_A$ Receptors Containing α5 Subunits Contributing to the Sedative Properties of Benzodiazepine Site Agonists? Neuropsychopharmacology.

Scimemi A, Semyanov A, Sperk G, Kullmann DM, Walker MC (2005) Multiple and plastic receptors mediate tonic $GABA_A$ receptor currents in the hippocampus. J Neurosci 25:10016-10024.

Scoville WB, Milner B (2000) Loss of recent memory after bilateral hippocampal lesions. 1957. J Neuropsychiatry Clin Neurosci 12:103-113.

Semyanov A, Walker MC, Kullmann DM (2003) GABA uptake regulates cortical excitability via cell type-specific tonic inhibition. Nat Neurosci 6:484-490.

Semyanov A, Walker MC, Kullmann DM, Silver RA (2004) Tonically active $GABA_A$ receptors: modulating gain and maintaining the tone. Trends Neurosci 27:262-269.

Serwanski DR, Miralles CP, Christie SB, Mehta AK, Li X, De Blas AL (2006) Synaptic and nonsynaptic localization of $GABA_A$ receptors containing the α5 subunit in the rat brain. J Comp Neurol 499:458-470.

Shen H, Gong QH, Aoki C, Yuan M, Ruderman Y, Dattilo M, Williams K, Smith SS (2007) Reversal of neurosteroid effects at α4β2δ $GABA_A$ receptors triggers anxiety at puberty. Nat Neurosci 10:469-477.

Sieghart W, Sperk G (2002) Subunit composition, distribution and function of $GABA_A$ receptor subtypes. Curr Top Med Chem 2:795-816.

Sigel E, Schaerer MT, Buhr A, Baur R (1998) The benzodiazepine binding pocket of recombinant α1β2γ2 $GABA_A$ receptors: relative orientation of ligands and amino acid side chains. Mol Pharmacol 54:1097-1105.

Singer W (1999) Neuronal synchrony: a versatile code for the definition of relations? Neuron 24:49-65, 111-125.

Singer W, Kreiter AK, Engel AK, Fries P, Roelfsema PR, Volgushev M (1996) Precise timing of neuronal discharges within and across cortical areas: implications for synaptic transmission. J Physiol Paris 90:221-222.

Somogyi P, Fritschy JM, Benke D, Roberts JD, Sieghart W (1996) The γ2 subunit of the $GABA_A$ receptor is concentrated in synaptic junctions containing the α1 and β2/3 subunits in hippocampus, cerebellum and globus pallidus. Neuropharmacology 35:1425-1444.

Somogyi P, Klausberger T (2005) Defined types of cortical interneurone structure space and spike timing in the hippocampus. J Physiol 562:9-26.

Sperk G, Schwarzer C, Tsunashima K, Fuchs K, Sieghart W (1997) $GABA_A$ receptor subunits in the rat hippocampus I: immunocytochemical distribution of 13 subunits. Neuroscience 80:987-1000.

Stein V, Nicoll RA (2003) GABA generates excitement. Neuron 37:375-378.

Stell BM, Brickley SG, Tang CY, Farrant M, Mody I (2003) Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subunit-containing $GABA_A$ receptors. Proc Natl Acad Sci U S A 100:14439-14444.

Stell BM, Mody I (2002) Receptors with different affinities mediate phasic and tonic $GABA_A$ conductances in hippocampal neurons. J Neurosci 22:RC223.

Sun C, Sieghart W, Kapur J (2004) Distribution of α1, α4, γ2, and δ subunits of $GABA_A$ receptors in hippocampal granule cells. Brain Res 1029:207-216.

Sundstrom-Poromaa I, Smith DH, Gong QH, Sabado TN, Li X, Light A, Wiedmann M, Williams K, Smith SS (2002) Hormonally regulated α4β2δ $GABA_A$ receptors are a target for alcohol. Nat Neurosci 5:721-722.

Sur C, Wafford KA, Reynolds DS, Hadingham KL, Bromidge F, Macaulay A, Collinson N, O'Meara G, Howell O, Newman R, Myers J, Atack JR, Dawson GR, McKernan RM, Whiting PJ, Rosahl TW (2001) Loss of the major $GABA_A$ receptor subtype in the brain is not lethal in mice. J Neurosci 21:3409-3418.

Szabadics J, Varga C, Molnar G, Olah S, Barzo P, Tamas G (2006) Excitatory effect of GABAergic axo-axonic cells in cortical microcircuits. Science 311:233-235.

Tamas G, Lorincz A, Simon A, Szabadics J (2003) Identified sources and targets of slow inhibition in the neocortex. Science 299:1902-1905.

Tauber M, Calame-Droz E, Prut L, Rudolph U, Crestani F (2003) α2- $GABA_A$ receptors are the molecular substrates mediating precipitation of narcosis but not of sedation by the combined use of diazepam and alcohol *in vivo*. Eur J Neurosci 18:2599-2604.

Thomson AM, Bannister AP, Hughes DI, Pawelzik H (2000) Differential sensitivity to Zolpidem of IPSPs activated by morphologically identified CA1 interneurons in slices of rat hippocampus. Eur J Neurosci 12:425-436.

Tobler I, Kopp C, Deboer T, Rudolph U (2001) Diazepam-induced changes in sleep: role of the α1-$GABA_A$ receptor subtype. Proc Natl Acad Sci U S A 98:6464-6469.

Toledo-Rodriguez M, Blumenfeld B, Wu C, Luo J, Attali B, Goodman P, Markram H (2004) Correlation maps allow neuronal electrical properties to be predicted from single-cell gene expression profiles in rat neocortex. Cereb Cortex 14:1310-1327.

Towers SK, Gloveli T, Traub RD, Driver JE, Engel D, Fradley R, Rosahl TW, Maubach K, Buhl EH, Whittington MA (2004) α5 subunit-containing $GABA_A$ receptors affect the dynamic range of mouse hippocampal kainate-induced γ frequency oscillations in vitro. J Physiol 559:721-728.

Tretter V, Ehya N, Fuchs K, Sieghart W (1997) Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. J Neurosci 17:2728-2737.

van Rijnsoever C, Tauber M, Choulli MK, Keist R, Rudolph U, Mohler H, Fritschy JM, Crestani F (2004) Requirement of α5-$GABA_A$ receptors for the development of tolerance to the sedative action of diazepam in mice. J Neurosci 24:6785-6790.

Verdoorn TA (1994) Formation of heteromeric $GABA_A$ receptors containing two different α subunits. Mol Pharmacol 45:475-480.

Vicini S, Ferguson C, Prybylowski K, Kralic J, Morrow AL, Homanics GE (2001) $GABA_A$ receptor α1 subunit deletion prevents developmental changes of inhibitory synaptic currents in cerebellar neurons. J Neurosci 21:3009-3016.

Wallner M, Hanchar HJ, Olsen RW (2003) Ethanol enhances α4β3δ and α6β3δ $GABA_A$ receptors at low concentrations known to affect humans. Proc Natl Acad Sci U S A 100:15218-15223.

Wei W, Zhang N, Peng Z, Houser C, Mody I (2003) Perisynaptic Localization of α-Subunit-Containing $GABA_A$ receptors and their activation by GABA spillover in the mouse dentate gyrus. The Journal of Neuroscience:1014– 1021.

Whiting PJ (2003) The $GABA_A$ receptor gene family: new opportunities for drug development. Curr Opin Drug Discov Devel 6:648-657.

Whittington MA, Traub RD (2003) Interneuron diversity series: inhibitory interneurons and network oscillations *in vitro*. Trends Neurosci 26:676-682.

Wieland HA, Luddens H, Seeburg PH (1992) A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. J Biol Chem 267:1426-1429.

Williams SR, Stuart GJ (2003) Voltage- and site-dependent control of the somatic impact of dendritic IPSPs. J Neurosci 23:7358-7367.

Wohlfarth KM, Bianchi MT, Macdonald RL (2002) Enhanced neurosteroid potentiation of ternary $GABA_A$ receptors containing the δ subunit. J Neurosci 22:1541-1549.

Yamada J, Furukawa T, Ueno S, Yamamoto S, Fukuda A (2006) Molecular Basis for the $GABA_A$ Receptor-Mediated Tonic Inhibition in Rat Somatosensory Cortex. Cereb Cortex.

Yee BK, Hauser J, Dolgov VV, Keist R, Mohler H, Rudolph U, Feldon J (2004) GABA receptors containing the α5 subunit mediate the trace effect in aversive and appetitive conditioning and extinction of conditioned fear. Eur J Neurosci 20:1928-1936.

Yeung JY, Canning KJ, Zhu G, Pennefather P, MacDonald JF, Orser BA (2003) Tonically activated $GABA_A$ receptors in hippocampal neurons are high-affinity, low-conductance sensors for extracellular GABA. Mol Pharmacol 63:2-8.

Acknowledgements

I would like to thank Prof. Jean-Marc Fritschy for his excellent supervision and support. I very much appreciate his objective and pragmatic way of guiding me during this thesis giving me enough freedom and trust to decide how to direct my work. The respect of Jean-Marc towards his group creates a very good working atmosphere and his artistic and scientific passion revealing the structure of the Brain allowed me to see science from a unique perspective.

Many thanks to Prof. Dr. Kaspar Vogt for giving me the opportunity to initiate my PhD in electrophysiology; to my 'Doktorvater' Prof. Peter Sonderegger and my supervisor Prof. Beat Gähwiler, for their agreement on evaluating this thesis; to Barbara Ellis for proof reading my work and to Christina Koster for correcting the 'Zusammenfassung'.

A sincere thank goes to Corinne Sidler, who introduced me into the technique of immunofluorescence staining and together with me created the so called 'Method 4'; she also helped me during my pregnancy with the DAB stainings and the paraformaldehyde steps; without her it would have been impossible to go on. Thanks as well to Franziska Parpan and Ruth Keist for breeding and genotyping the mice.

I would like to tank in particular my working colleagues George Prenosil, Carolin Straub and Venceslas Duveau for collaborating on my projects. Also thanks to Ela Balic and Christine Gee for their coffee breaks with exciting scientific discussions, and to the rest of my colleagues Laetitia Gabernet, Caroline Petitjean, Barbara Lardi, Shiva Tiagarajan, Martina Baldinger, Anja Zeller, Marija Simat and Jorge Soliz for the good friendship and cooperation.

Finally my special thanks go to all the people who helped me to make this 'Baby-PhD' possible. First of all to my husband Stefan for his constant support and for the uncountable evenings he took care of Sebastian. Thanks also to Annelies and Kathrin for taking care of Sebastian all these years and to Laura to take care of Martín during these last months.

Curriculum Vitae

BIOGRAPHICAL DATA

Born:	22 July 1976 in Zürich
Nationality:	Swiss: Seeberg (Bern) and Bolivian
Civil status:	married
Children:	two (26.01.02 and 09.12.06)

EDUCATION

1990 – 1994	Colegio San Ignacio, La Paz – Bolivia
	Bachillerato en humanidades
1995 – 6/2000	Universidad Mayor de San Andrés (UMSA), La Paz – Bolivia
	B.S. Biology
7/2000 – 6/2001	University of Zürich, Switzerland
	Diploma thesis. Advisor: Dr. Markus U. Ehrengruber.

`Overexpression of connexin 36 (Cx-36) in neurons from cultured hippocampal slices by using recombinant semliki forest virus`

9/2001	Universidad Mayor de San Andrés (UMSA), La Paz - Bolivia
	Diploma degree in Biology
1/2002 - 9/2002	Maternal leave
10/2002	Exam "Biochemistry Techniques" requested to enter the PhD program
2003 - 2006	PhD student in neurobiology, Institute of Pharmacology & Toxicology, Zürich University, Switzerland. 'Functional role and plasticity of $GABA_A$ receptors in the mouse hippocampus'

TEACHING EXPERIENCE

1996 – 2000 **Private teacher** in physics at level of gymnasium

Teaching assistant, Faculty of natural Science, University of La Paz – Bolivia. Supervising ungraduated students in the following laboratory courses.

7/1997 – 12/1997 Non-vascular plants botany (20 hours/month)

1998 Biophysics (20 hours/month)

2/1999 – 6/1999 Microbiology (20 hours/month)

2/2000 - 6/2000 Animal physiology (20 hours/month)

2004 - 2006 **Teaching assistant,** Zürich University. Supervising biology students in practical courses in Biochemistry (4 h/weekly each second semester)

SPECIAL SKILLS

Laboratory techniques: Molecular biological techniques (subcloning and construction of viral vectors); immunohistochemistry (immunostaining in tissue sections); electrophysiology (whole-cell patch-clamp).

Microscopy: Bright field, fluorescence and confocal laser scanning.

LANGUAGES

Spanish mother tongue

English speak, write and read fluently

German good understanding

Publications

1. Lundstrom K., Rotmann D., Hermann D., **Schneider E.M.,** Ehrengruber M.U. (2001) Novel mutant Semliki Forest virus vectors: gene expression and localization studies in neuronal cells. *Histochem Cell Biol.* 115, 83-91.

2. Lundstrom K, Schweitzer C, Rotmann D, Hermann D., **Schneider E.M.**, Ehrengruber M.U. (2001) Semliki forest virus vectors: efficient vehicles for in vitro and *in vivo* gene delivery. *FEBS Letters* 504, 99-103.

3. Hennou S, Kato A, **Schneider E.M.**, Lundstrom K, Gähwiler BH, Inokuchi K, Gerber U, Ehrengruber M.U. (2003) Homer-1a/Vesl-1S enhances hippocampal synaptic transmission. *Eur J Neurosci*. Aug:18(4):811-819.

4. Prenosil, G.A.*, **Schneider Gasser E.M.***, Rudolph U., Keist R, Fritschy J.-M. and Vogt K.E. (2006*)* Specific subtypes of $GABA_A$ receptors mediate phasic and tonic forms of inhibition in hippocampal pyramidal neurons. *J Neurophysiol* 96(2):846-857.
* First co-authorship

5. **Schneider Gasser E.M**.*, Straub C.J.*, Panzanelli P, Weinmann O., Sassoè-Pognetto M., Fritschy J.-M. (2006) Immunofluorescence in brain sections: simultaneous detection of presynaptic and postsynaptic proteins in identified neurons. *Nat Protoc*. 1(4):1887-1897. * First co-authorship

6. **Schneider Gasser E.M**, Duveau V., Prenosil G.A., Fritschy J.-M. (2007) Reorganization of GABAergic circuits maintains $GABA_A$ receptor-mediated transmission onto CA1 interneurons in α1 subunit-null mice. *Eur J Neurosci*. In press.